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(54) Title: ISOLATED ANTIGENIC ONCOGENE PEPTIDE FRAGMENTS AND USES			
(57) Abstract <p>An isolated antigenic peptide fragment that is an isolated oncogene protein fragment is disclosed. The isolated peptide fragment of the invention is a peptide of either 9 or 10 amino acid residues in length that includes at least a leucine residue at the C-terminus (i.e., position 9 or position 10). The peptide fragment is capable of binding in an HLA-A2 binding cleft and is capable of stimulating proliferation of at least one tumor-specific cytotoxic T-lymphocyte. Preferred peptides further include an isoleucine residue at position 2 and a valine residue at position 6. The most preferred isolated peptides can stimulate proliferation of cytotoxic T-lymphocytes obtainable from peripheral blood lymphocytes, ovarian tumors, breast tumors, gastric tumors, non-small cell lung tumors, pancreatic tumors, colon tumors, gliomas, bladder tumors, endometrial tumors and neuroblastomas. The preferred isolated peptide of the invention is a mutant peptide defined by SEQ ID : 2 or its functional equivalents. Isolated cytotoxic T-lymphocytes capable of recognizing isolated peptides of the invention, particularly a HER2/neu mutant peptide are disclosed. These lymphocytes are especially useful for killing tumors or other cells which present on their surfaces the antigenic peptides of the invention. In particular, the lymphocytes are obtainable from ovarian tumor cells, breast tumor cells and non-small-cell lung tumor cells. A method of stimulating proliferation of tumor-specific cytotoxic T-lymphocytes is described. This method includes obtaining lymphocytes from tumor tissue and contacting the lymphocytes with a peptide of the invention under conditions sufficient for the cytotoxic T-lymphocytes to proliferate. Therapeutically effective compositions containing the HER2/neu peptides are also described.</p>			

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Isolated Antigenic Oncogene Peptide Fragments and Uses

Background of the Invention

The immune system plays a central role in the body's defense against cancer. Cellular immunity against tumors can be demonstrated in part because the ability of T-lymphocytes to engage in cell-mediated attack against tumor cells is made possible by the interaction between the T-lymphocyte receptor ("TCR": T- cell receptor) and specific class 1 HLA tumor cell markers (i.e., human leukocyte antigens: "HLA" molecules) present on the surface of the tumor cell. The TCR's on T-lymphocytes recognize a tumor antigen when the tumor antigen is associated with the HLA cell surface molecules. In particular, CD8 T-lymphocytes recognize antigens made within, or otherwise introduced into, the cytoplasm of the cell. Antigens in the cells are cleaved by cytoplasmic proteases into peptide fragments. The fragments are transported by an active transport process mediated by molecules encoded in the HLA itself into the endoplasmic reticulum where the fragments bind to newly made class I molecules. The peptide fragment is transported out to the cell surface where the T-lymphocytes recognize the combination of peptide and HLA. The peptide binds in the groove of the HLA molecule between two alpha helices that form the sides of the groove.

Through this recognition of presented surface tumor antigen (i.e., tumor-associated antigen: "TAA"), the T-lymphocytes are activated, expanded by clonal selection, and matured to give rise to tumor-specific, cytotoxic T-effector cells ("cytotoxic T-lymphocytes") together with an enlarged population of T-memory cells. Unfortunately, tumor cells are not especially effective in mediating strong immune responses.

The identity of the TAA's themselves is generally unknown. Recently, TAA's have been investigated in two different tumor systems. It has been demonstrated that T-lymphocytes recognize specific TAA presented by HLA moieties on the surface of melanoma cells; the so-called

5 MAGB system. See for example, Kawakami et al., J. Immunol., 148:638, (1992). The MAGB system is, however, expressed in only 40-50% of these tumors which make up only about 1 percent of all cancers. The MZB-E antigenic peptide of the MAGB system is presented by HLA-A1 molecules which are found in only 26% of Caucasians. Specific recognition of ovarian cancer TAA by T-lymphocytes has also been demonstrated. See Ioannidis et al., Cel1 Immuno1. 151:225-234 (1993), incorporated herein by reference.

10 A source of the ovarian cancer TAA may be fragments of oncofetal proteins. HRR2/neu (also called c-erbB-2) is a 185 kDa transmembrane glycoprotein which tyrosine kinase activity and extensive homology to epidermal growth factor. HRR2/neu is a ubiquitous oncofetal factor. HRR2/neu is expressed in normal cells (Cousens et al., Science, 230: 1132) and overexpressed in about 30% of all ovarian and breast cancers. Overexpression of the HRR2/neu oncogene correlates with a poor prognosis in these cancer patients. Slamon et al., U.S. Patent No. 4,968,603 (Nov. 6, 1990), incorporated herein by reference.

15 The identity of specific antigenic peptide fragments which are the target of T-lymphocyte recognition would allow peptides to be used to stimulate T-lymphocytes in vitro for specific adoptive immunotherapy.

20 Nonetheless, although HRR2/neu is widely expressed in epithelial tumors such as ovarian, breast, lung, gastric, pancreatic, and colon (See, for example, Yamamoto et al., Human Pathology, 24:1127-1134 (1993); Kern et al., Am. J. Resp. Cel1 Mol. Biol. 9:448-454 (1993)), only peptide fragments based on HRR2/neu proteins from normal cells have heterologous to provide recognition for cytotoxic T-lymphocytes. See Ioannidis et al., Abstract PASB Meeting, 1992 page A1404. Furthermore, because of their amino acid sequence and size, these "normal" HRR2/neu peptide fragments are not actually predicted to bind with HLA-A2 molecules on the tumor surface and therefore would

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not be presented on the cell surface for immune recognition.

Summary of the Invention

The present invention pertains to an isolated antigenic peptide fragment from tumor cells that is an isolated 5 oncogene protein fragment.

An isolated peptide fragment of the invention is a peptide of either 9 or 10 amino acid residues in length that includes at least a leucine residue at the C- terminus (i.e., position 9 or position 10). The peptide fragment is 10 capable of binding in an HLA-A2 binding cleft and is capable of stimulating proliferation of at least one cytotoxic T-lymphocyte. Preferred peptides further include an isoleucine residue at position 2 and a valine residue at 15 position 6. The most preferred isolated peptides can stimulate proliferation of cytotoxic T-lymphocytes obtainable from peripheral blood lymphocytes, ovarian tumors, breast tumors, gastric tumors, non-small cell lung tumors, pancreatic tumors, colon tumors, gliomas, bladder 20 tumors, endometrial tumors and neuroblastomas.

In a preferred embodiment, the antigenic peptides are derived from a portion of the mutant HER2/neu protein found in tumor cells and not found in normal cells. This preferred isolated peptide of the invention is a mutant 25 peptide with an amino acid sequence defined by SEQ ID NO.:2 or its functional equivalents.

An isolated nucleic acid sequence of the invention is a nucleic acid sequence capable of encoding a peptide fragment of either 9 or 10 amino acid residues in length, 30 the peptide fragment including at least a leucine residue at a C-terminus and capable of engaging with an HLA-A2 binding cleft. The encoded peptide is also capable of stimulating proliferation of at least one cytotoxic T-lymphocyte when the peptide is bound in an HLA-A2 binding 35 cleft. Preferred nucleic acid sequences are capable of encoding a mutant HER2/neu peptide fragment (e.g., SEQ ID

NO.:2) that is of sufficient size and amino acid composition to engage with an HLA-A2 binding peptide. Another aspect of the invention is a recombinant vector including the isolated cytotoxic T-lymphocytes capable of recognizing the isolated peptide of the invention, particularly the HLA-A2 mutant peptide, when the peptide is engaged with an HLA-A2 binding peptide, wherein the peptide is obtained from peripheral blood lymphocytes, the HLA-A2 mutant peptide is obtained from the invention, in which surfaces or other cells which present on non-smooth cells lung tumor cells, ovarian tumor cells, breast tumor cells, colon tumors, gliomas, bladder tumors, endometrial tumors, neurorhabdomas and gastric tumors.

The invention further embodies a method of stimulating proliferation of tumor-specific cytotoxic T-lymphocytes. This method includes obtaining T-lymphocytes from tumor tissue and contacting the T-lymphocytes from tumor tissue and contacting the T-lymphocytes with a cytotoxic T-lymphocyte to proliferate. Preferably, the peptide of the invention under conditions wherein it is engaged with an HLA-A2 peptide contacts with a mutant HLA-A2 peptide to engage with it.

A composition is also described. A preferred composition includes a cytotoxic T-lymphocyte to engage with an HLA-A2 peptide of sufficient size and amino acid banding cleavage. The composition may also include an antigen-binding fragment. This particulate composition is presented in a combination with the anti-idiotype peptide. The composition may also include an antibody combining fragment.

Another aspect of the invention is an in vitro proliferation of tumor cells. This is particularly useful in an anti-tumor agent.

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It is an object of the present invention to provide an antigenic peptide capable of binding with the HLA-A2 binding cleft.

5 It is a further object of the present invention to provide an antigenic peptide capable of binding with the HLA-A2 binding cleft that stimulates proliferation of cancer-specific CTL's and induces cytotoxicity and/or cytokine release.

10 It is another object of the invention to provide a means for stimulating proliferation of cytotoxic T-lymphocytes useful for killing tumors (i.e., ovarian tumors, breast tumors, gastric tumors, non-small cell lung tumors, pancreatic tumors, colon tumors, gliomas, bladder tumors, endometrial tumors and neuroblastomas) or other 15 cells which present on their surfaces the antigenic peptides of the invention.

Detailed Description of the Drawings

20 Figure 1 illustrates results of chromium release assays that demonstrate OvTIL recognition of HLA-A2⁺, HER2/neu⁺ breast cancer lines. Three HLA-A2⁺ ovarian tumor-specific CTL lines were tested against HLA-A2⁺ and HLA-A2⁻, HER2/neu⁺ breast cancer lines in cytotoxicity assays at an E:T ratio of 20:1.

25 Figure 2 illustrates results of chromium release assays that demonstrate BrTIL recognition of HLA-A2⁺, HER2/neu⁺ ovarian cancer lines. Three HLA-A2⁺ breast tumor-specific CTL lines were tested against HLA-A2⁺ and HLA-A2⁻, HER2/neu⁺ ovarian cancer lines in cytotoxicity assays at an E:T ratio of 20:1.

30 Figure 3 illustrates results of chromium release assays that demonstrate ovarian tumor-specific CTL's recognize the HER2/neu-derived peptide (SEQ ID NO.: 2). OvTIL were tested against T2 either unloaded or pulsed with 50-100 ug of SEQ ID NO.1 or 2 at an E:T ratio of 20:1.

35 Figure 4 illustrates results of chromium release assays that demonstrate breast tumor-specific CTL's recognize the

5 Figure 5 illustrates that OTII recognizes SRQ ID NO.: 2 in a dose response manner. OTII were tested against T2 either unloaded or pulsed with 50-100 μ g of SRQ ID NO. 1 or 2 at an R:T ratio of 20:1. R:T of 20:1 and peak recognition occurred at 100 μ g/ml. Figure 6 illustrates that recognition of SRQ ID NO.: 2 (GP2). Incubations were for 1 hour at an R:T of 20:1 and peak recognition occurred at 100 μ g/ml. Figure 6 illustrates that recognition of SRQ ID NO.: 2 at 100 μ g/ml was chromatin labelled, (GP2) is HLA-A2 restricted. T2 was chromatin labelled, pulsed with SRQ ID NO.: 2, and pulsed with monoclonal antibodies added at 1:2 dilution of hybridoma supernatant for 30 minutes at 37 degrees C prior to chromatin release assays. The data presented is for OTII and is represented in figure 7 illustrates results of chromatin release assays that demonstrate non small-cell lung cancer-specific CTL's recognize the HRR2/neu-derived peptide (SRQ ID NO.: 2). TII, s were tested against T2 either unloaded or pulsed with 50-100 μ g of SRQ ID NO. 1 or 2 at an R:T ratio of 20:1. Figure 8 illustrates results of chromatin release assays that demonstrate ovarian cancer T-lymphocytes that have been educated with SRQ ID NO.: 2 recognize HLA-A2+, HRR2/neu+ allogenic tumors. T-lymphocytes were tested against T2 targets loaded with 50-100 μ g of SRQ ID NO. 1 (bar 1) or SRQ ID NO.: 2 (bar 2), a2 positive ovarian tumor targets (bars 3 and 4), and an A2 negative ovarian tumor target (bar 5), all at an R:T ratio of 20:1.

20 Figure 7 illustrates results of multiple assays performed that demonstrate non small-cell lung cancer-specific CTL's recognize the HRR2/neu-derived peptide (SRQ ID NO.: 2). TII, s were tested against T2 either unloaded or pulsed with 50-100 μ g of SRQ ID NO. 1 or 2 at an R:T ratio of 20:1.

25 Figure 8 illustrates results of chromatin release assays that demonstrate T2 targets loaded with 50-100 μ g of SRQ ID NO. 1 against HRR2/neu+ allogenic tumors. T-lymphocytes were tested against T2 targets (bars 3 and 4), and an A2 negative ovarian tumor targets (bar 5), all at an R:T ratio of 20:1.

30 Description of the sequences SRQ ID NO.: 1 is the wild-type HRR2/neu peptide fragment SRQ ID NO.: 2 is a mutant HRR2/neu peptide fragment (HLA Leu Ser Ala Val Glu HLe Leu).

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SEQ ID NO.:3 is the canonical HER2/neu sequence containing anchor binding residues to the HLA-A2 cleft (Xaa Ile Xaa Xaa Xaa Val Xaa Xaa Leu);

5 SEQ ID NO.:4 is HER2/neu peptide that does not induce proliferation of cancer-specific CTL's (Pro Leu Thr Ser Ile Ile Ser Ala Val);

10 SEQ ID NO.:5 is a functional variant of SEQ ID NO.:2 (Tyr Ile Ser Ala Val Val GLy Ile Leu);

SEQ ID NO.:6 is a functional variant of SEQ ID NO.:2 (Phe Ile Ser Ala Val Val Gly Ile Leu);

15 SEQ ID NO.:7 is a nucleic acid sequence encoding SEQ ID NO.:2; (ATCATCTCTGCCTGGTGGCATTCTG)

20 SEQ ID NO.: 8 is a fully degenerate oligonucleotide capable of encoding SEQ ID NO.:2; (ATHATHAGYGCNGTNGTNGGNATHTTR)

25 SEQ ID NO.: 9 is a fully degenerate oligonucleotide capable of encoding SEQ ID NO.:2; (ATHATHTCGCNGTNGTNGGNATHTTR)

SEQ ID NO.: 10 is a fully degenerate oligonucleotide capable of encoding SEQ ID NO.:2; (ATHATHAGYGCNGTNGTNGGNATHCTN)

30 SEQ ID NO.: 11 is a fully degenerate oligonucleotide capable of encoding SEQ ID NO.:2; (ATHATHTCGNGCNGTNGTNGGNATHCTN)

35 SEQ ID NO.: 12 is a peptide used in the A2.1 binding assay; Phe Leu Pro Ser Asp Tyr Phe Pro Ser Val.

below).

35 containing nucleotide sequences encoding the peptide (see sequences encoding the peptide or from a recombinant vector invention including peptide expressed from nucleotide not occur in nature. Isolated antigenic peptides of the peptide in its naturally-occurring state; or (v) that do the like) other than those associated with the antigenic motives (e.g. peptides, carbohydrates, fatty acids, and analytical procedures; or (iv) associated with chemical biologically active acids using purified using protein methods; or (ii) separated from naturally-occurring (i); synthesized by, for example, chemical synthesis; or encoded by nucleic acids using recombinant DNA methods; or peptides of the present invention means peptides: (i)

25 The term "isolated", when applied to the antigenic peptides of the lymphocytes agglutinates the peptide. from B-lymphocytes human immune responses (i.e., proliferation of CTLs) and/or stimulate responses (i.e., proliferation of CTLs) and/or stimulate peptides of the invention to stimulate cell-mediated immune the term "antigenic" refers to the ability of the release by the lymphocytes.

20 The chromatin release assay) and/or induction of cytotoxicity as induced by cytotoxicity (as measured by, for example, encompasses other functional characteristics of CTLs, such include growth and clonal selection of CTLs, and release by the lymphocytes.

25 The chromatin release assay) and/or induction of cytotoxicity as induced by cytotoxicity (as measured by, for example, encompasses other functional characteristics of CTLs, such include growth and clonal selection of CTLs, and release by the lymphocytes.

30 The peptide is recognized by cancer-specific CTLs of different origins and stimulates proliferation of cancer-interlocak or mesh together. The term "engagé" has its ordinary meaning of to molecule. The peptide to engage the binding cleft of an HLA-A2 acid composition to that is of sufficient size and amino acid an oncogene and that is a portion of the protein product peptide fragment that is isolated, antigenic

5 One aspect of the invention is an isolated, antigenic

A. ISOLATED PEPTIDE SEQUENCES

Descriptive of the invention

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Preferred antigenic peptides of the invention are gene product fragments of the HER2/neu oncogene (also called c-erb2). See Coussens et al. *supra* for sequence of the HER2/neu protein, incorporated herein by reference.

5 HER2/neu protein is expressed in normal tissues but is also amplified and over-expressed in tumor cells.

Because the HER2/neu peptides of the invention are particularly useful in initiating tumor-specific CTL proliferation (see Example 5), initiation of CTL action 10 against normal, host cells and tissues is of concern.

Preferred peptides of the invention are derived from tumor cells and are referred to as "mutant" peptides (i.e., expressed in tumor cells and tissues). The term "mutant" 15 also refers to the fact that many peptides of the invention have one or more point mutations in their amino acid sequence as compared to "wild type" (i.e., normal) sequences.

Mutant peptides are preferentially associated with tumor tissue relative to normal tissues. For example, 20 oncogene products are often overexpressed in tumor tissues, but not in normal (i.e. non-tumor) tissues. Peptides derived from such overexpressed oncogene products would be more likely to be presented on the surface of a tumor cell than on the surface of a normal cell and would therefore be 25 "preferentially associated" with tumor tissue.

Particularly preferred are antigenic peptides that are 30 associated only with tumor tissue. For example, as is the case with HER2/neu, oncogene products produced in tumor tissues sometimes bear mutations not found in non-tumor tissues. In some instances, mutant oncogene products will contain peptides that can be presented in association with an HLA-A2 molecule, and that are not found in the 35 corresponding "normal" oncogene product. Such mutant peptides would therefore be associated only with tumor tissues and, like the mutant HER2/neu peptide described above, represent particularly preferred embodiments of the present invention.

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5 Preferred antigenic peptides described herein are the gene product fragments of oncogenes expressed in a variety of tumor cells. In the case of the HER2/neu oncogene, it is expressed in many epithelial tumors, including breast (Slamon et al.; Science, 244: 707-712, 1989), ovarian (Tornand, et al., Cell, Immuno, 151:225, 1993), and gastric (Yamamoto et al., Nature, 319:230, 1986).

10 HER2/neu antigenic peptides described herein, when presented by HLA-A2 molecules, are recognized by cancer-specific cytotoxic T lymphocytes from many different tumors. Thus, the preferred antigenic peptides of the tumor may be widely recognized since 50% of the tumors express HLA-A2, and many different tumors express a fragment of the HER2/neu oncogene protein involved in a point mutation found in the transmembrane portion of the tumor-derived HER2/neu protein and is not expressed in normal tissue.

15 20 Preferred antigenic peptides bound to human major histocompatibility complex (MHC) class I molecules is a preaguistic for cytotoxic T lymphocyte activation of cytotoxic T lymphocyte reactions and cytolytic release. See Examples 4 and 5. The structure of the antigenic peptides are therefore strictly constrained by the requirement that they engage with the binding cleft of the histocompatibility antigen.

25 30 Leukocyte antigen (HLA), HLA-A2.

1. HLA-A2 Binding Requirements

2. HLA-A2 Binding Requirements

35 Certain binding requirements for HLA-A2 are well characterized. Recent studies show that the majority of peptides binding to MHC class I molecules, in particular HLA-A2 molecules, have a size of 9-10 amino acids and "fragments" of the invention is no greater than 10 amino acids long and is most preferably 9 amino acids long.

reguire free N- and C-terminal ends. Thus, a peptide HLA-A2 molecules, have a size of 9-10 amino acids and peptides binding to MHC class I molecules, in particular HLA-A2 molecules, have a size of 9-10 amino acids and characterize free N- and C-terminal ends. Thus, a peptide

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Amino acid sequences longer than 10-mer (see for example, Ioannides, *supra*) will likely not engage the binding cleft at all. In addition to this size constraint, the HLA-A2 binding cleft requires two aliphatic hydrophobic anchor residues within the peptide ligand, these being leucine (L) at position 2 and L or valine (V) at the C-terminal end. See Falk et al., *Nature*, 351:290-296, 1991, incorporated herein by reference.

The role of nonanchor residues in determining HLA-A2 binding has recently been investigated. See Ruppert et al., *Cell*, 71:929-937, 1993, incorporated herein by reference. Amino acid residues strongly associated with good binding by HLA-A2 have been analyzed so that the number and sequence of possible 9-mer and 10-mer antigenic peptides of the invention is further constrained within narrow limits. The following Table, adapted from Ruppert et al., *id*, lists those amino acid residues associated with good binding of any 9-mer and 10-mer peptides to the binding cleft of HLA-A2.

9-Mer Peptides									
POSITION:									
1	2	3	4	5	6	7	8	9	
25	Y	L,M	Y	S	Y	A	P	L,V,I	
	F		F	T	F				
	W		W	C	W				

10-Mer Peptides									
POSITION:									
1	2	3	4	5	6	7	8	9	10

20 When the transmembrane region (amino acids 654 to 662) of the human HR2/neu protein from breast cancer tissue was 7:2043, 1988.

25 In the transmembrane portion of the corresponding rat neu gene there is a single point mutation (glutamic acid for valine) at residue 659 which enables this rat gene to transform the cell. See Bargmann et al. RMB 31.

30 When the transmembrane region (amino acids 654 to 662) of the human HR2/neu protein from breast cancer tissue was 7:2043, 1988.

35 This substitution was also found by Diflore et al. Yamamoto et al. (Nature: 319:230-234, 1986) described a gastric cancer-derived full-length sequence that also contained the substitution at position 655. Therefore, it appears that the HR2/neu protein expressed in cancer tissue or cell lines contains the mutation at 655 as well.

10 Rupper et al., supra, also discusses which residues are not destrable at each position of an HLA-A2-binding peptide.

A	L, M	L	G	G	Y	V	I, V, I	Y	R
M		I							
I									
V									
L									
W									
F									

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compared to the wild-type sequence described by Coussens et al. derived from normal tissue.

We have discovered a correlation between HER2/neu expression and cytotoxic lymphocyte recognition by transfecting an HLA-A2+ melanoma with the HER2/neu gene rendering it recognizable to ovarian cancer-specific cytotoxic T-lymphocytes (CTL's). See Example 3.

In order to address the question of whether the HER2/neu is actually the source of the antigenic peptides recognized by the CTL's, the known amino acid sequences of HER2/neu (Coussens, et al., *supra*; Slamon et al., *supra*) were hand searched for nine-mer peptides containing the known canonical HLA-A2 position 2 and position 9 binding motifs.

Results of this search reveal that the known HER2/neu amino acid sequences from tumor cells all have the identical mutant peptide sequence, IISAVVGIL: SEQ ID NO.: 2. This mutant peptide has the HLA-A2 anchor binding residue basic structure xIxXXVxxL (SEQ ID NO.: 3) where I at position 2 is the mutation (HER2/neu position 655). This canonical sequence is not found between amino acids 654 and 662 of the wild-type HER2/neu oncogene protein (SEQ ID NO.: 1) and the normal peptide is not predicted to engage with the HLA-A2 binding cleft. See Coussens et al., *supra*.

Therefore, in cancer SEQ ID NO. 2 and 3 would be a potential site of recognition since this amino acid sequence would appear foreign to the immune system. The basic structure of SEQ ID NO.: 3 is identical to that for the influenza matrix peptide 58-66 (Bednarak et al., *J. Immunol.*, *supra*, incorporated herein by reference) which has been shown to bind the HLA-A2 molecule.

We constructed a synthetic peptide (SEQ ID NO.: 2: "GP2") and demonstrated recognition of this peptide by T-lymphocytes educated with autologous tumor (See Example 5).

Therefore, antigenic peptides of the invention include, but are not limited to, those containing as a primary amino

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sequence differs from the amino acid sequence depicted in SEQ ID NO.2 in that one or more of the amino acid residues of SEQ ID NO.2 has been substituted by another amino acid such that the relationship between the different amino acid sequences results in functional characteristics that are substantially the same. That is, a "functional equivalent" of SEQ ID NO.:2 will also elicit the identical qualitative T-lymphocyte responses of initiating CTL proliferation, cell cytotoxicity, and cytokine production. Substitutions of particular amino acids (See Table) at non-anchor 5 positions 1,3,4,5,6,7, and/or 8 of the 9-mer antigenic peptide of SEQ ID NO.:2 may be tested for functioning and may not produce radical changes in the physical and 10 chemical characteristics of the mutant peptide, in which case mutant peptides containing the substitution would be 15 considered to be functionally equivalent to peptides lacking the substitution. Functionally equivalent substitutes for an amino acid within the mutant HER2/neu antigenic peptide binding sequence of SEQ ID NO.: 2 may 20 therefore be selected from the Table. Significantly, the substitutions can be chosen for their effect on: (i) maintaining the structure of the peptide backbone in 25 the area of the substitution, for example, as a sheet or helical conformation; (ii) maintaining the charge or hydrophobicity of the molecule at the substitution position; or (iii) maintaining the bulk of the side chain.

When it is difficult to predict the exact effect of the substitution in advance of doing so, one skilled in the art 30 will appreciate that the effect can be evaluated using routine screening assays as described herein and known in the art. For example, HLA-A2 binding affinity may be tested by the procedures summarized in Example 1.

Similarly, antigenic properties may be tested by 35 routine methods. A change in the immunological character of an antigenic peptide of the invention, such as binding to a given antibody, can be measured by an immunoassay such

antigenic function. One approach is to displace the interactions are replaced with those that enhance the those in which nonselective residues causing adverse could be expected to produce increased antigenicity are peptide. Substitution in, for example, SEQ ID NO.: 2 that the original peptide is capable of eliciting antigenic stronger immune response than that of eliciting a to produce altered peptides that are capable of eliciting a 30 The antigenic peptides of the invention can be altered 35

5. Altered peptides

and prostate: 11/93).
Bank: 12/93; EMBL Model: 6/93; Vecbase: 8/87; TDDA: 7/92;
Protein: 11/93; SwissProt: 8/93; Gen Pept: 10/93; Gen package will scan the following databases (PIR-
with the canonical sequences. This particular software Computer Group, Sequence Analysts Software Package, version 7.2, 575 Sethylene Drive, Madison, WI, to identify peptides with the aid of a computer program such as Genetics example, known oncogene products can be scanned by eye, or (e.g., SEQ ID NO.: 3) can easily be identified. For In particular, peptides containing the canonical sequences general protocol for isolating any other oncogene-derived peptides described above allows the development of a The information provided on the HRS2/neu mutant 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100

described or known in the art.
otherwise behave like TAA's in other assays herein of the mutant peptides of SEQ ID NO.: 2 would be expected to in vitro or in vivo. For example, functional equivalents sequences, etc.). And/or functional characteristics analyzed reference sequence, the presence of unique amino acid by examining physical characteristics (e.g. homology to a equivalence of two peptide sequences can also be assessed as a competitive type immunoassay. The functional 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100

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peptide, identify the individual residues within the peptide that either bind to the HLA-A2 molecule cleft or interact with the TCR, and to determine which residues are just spacers whose role may be filled by many different 5 amino acids. See Berzofsky, "Epitope Selection and Design of Synthetic Vaccines", Ann. N.Y. Acad. Sci., 256-264 (1993), incorporated herein by reference.

For example, peptides of the invention that are capable of engaging with an HLA-A2 binding cleft might not engage 10 optimally with that cleft. As discussed above, Ruppert et al., supra, have identified desirable and non-desirable amino acid residues for each position of an HLA-A2-binding peptide. Also, the statistical data presented by Falk et al., supra, provides indications of which amino acid 15 residues are desirable and non-desirable at individual positions of an HLA-A2-binding peptide. Peptides of the invention that have the canonical HLA-A2-binding sequence, which specifies the amino acid residues allowed at the HLA-A2 anchor positions, might still have non-desirable residues at non-anchor positions, and therefore might not 20 elicit as potent an immune response as would a peptide with more desirable amino-acid residues at non-anchor positions.

One of ordinary skill in the art would readily 25 recognize that peptides of the invention could be altered, in that non-desirable amino acid residues at non-anchor positions could be substituted with desirable amino acids, as defined by Ruppert et al., supra, and Falk et al., supra, to create an altered peptide. One of ordinary skill 30 in the art will further recognize that, where the information presented by Falk et al. and Ruppert et al. is in conflict, the information presented by Ruppert et al. is more likely to be reliable since Ruppert et al. performed direct binding studies whereas Falk et al. performed only 35 statistical analyses of bound peptides.

Altered peptides could be tested for their ability to engage with an HLA-A2 binding cleft and/or to elicit an

5 recombinant nucleotide expression techniques or by chemical
 35 The peptides of the invention may be prepared by
 6. Synthesis and purification of peptides

30 incorporated herein by reference.
 (eds.) J.B. Coligan et al., J. Wiley & Sons, New York, NY,
 25 texts such as Current Protocols in Immunology, Vol. 2,
 defined property known in the art and presented in standard
 hydrophobicity of different amino acids is a well-
 35 Ruppert et al. as non-discriminatory at that position. The
 less-hydrophobic residue that has not been defined by
 one hydrophobic non-anchor residue is substituted with a
 the amino acid sequence of SEQ ID NO.:2 in that at least
 peptides that have an amino acid sequence that differs from
 Example 2). It may therefore be useful to make altered
 difficult to work with due to its hydrophobicity (see
 40 The HRR2/neu mutant peptide of SEQ ID NO.:2 is
 phenylalanine, or tryptophan.

20 with a different residue, preferably tyrosine,
 residue at position 5 of SEQ ID NO.:2 has been substituted
 arginine, lysine, or histidine; and/or (ii) the valine
 residue, preferably not aspartic acid, glutamic acid,
 25 of SEQ ID NO.:2 has been substituted with a different
 residue, that differs from the amino acid sequence of
 SEQ ID NO.:2 in that (i) the serine residue at position 3
 preferred altered peptides of the invention have an amino
 acid sequence that differs from the amino acid sequence of
 (a serine residue) and at position 5 (a valine residue).
 30 residues, as defined by Balak et al., supra, at position 3
 acids. SEQ ID NO.: 2 contains non-discriminatory amino acid
 non-anchor positions are replaced with discriminatory amino
 35 NO.:2 could be altered so that non-discriminatory acids at
 In particular, the mutant HRR2/neu peptide of SEQ ID
 original peptide.

5 proliferation of cytotoxic T-cells directed against the
 would be tested for their ability to stimulate
 disclosed herein. Most preferably, the altered peptides
 immune response against the original peptide by the methods

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synthesis using standard peptide synthesis techniques. For example, antigenic peptides of the invention can be produced, for example, by expressing cloned nucleotide sequences of the invention (see below). Alternatively, peptides of the invention can be generated directly from intact oncogene protein products. Peptides can be specifically cleaved by proteolytic enzymes, including, but not limited to, trypsin, chymotrypsin or pepsin. Each of these enzymes is specific for the type of peptide bond it attacks. Trypsin catalyzes the hydrolysis of peptide bonds whose carbonyl group is from a basic amino acid, usually arginine or lysine. Pepsin and chymotrypsin catalyze the hydrolysis of peptide bonds from aromatic amino acids, particularly tryptophan, tyrosine and phenylalanine.

Alternate sets of cleaved peptide fragments are generated by preventing cleavage at a site which is susceptible to a proteolytic enzyme. For example, reaction of the ϵ -amino groups of lysine with ethyltrifluorothioacetate in mildly basic solution yields a blocked amino acid residue whose adjacent peptide bond is no longer susceptible to hydrolysis by trypsin. Goldberger et al. Biochem., 1:401 (1962).

Peptides of the invention also can be modified to create peptide linkages that are susceptible to proteolytic enzyme catalyzed hydrolysis. For example, alkylation of cysteine residues with 6-halo ethylamines yields peptide linkages that are hydrolyzed by trypsin. Lindley, Nature, 178: 647 (1956). In addition, chemical reagents that cleave peptide chains at specific residues can be used. Withcop, Adv. Protein Chem. 16: 221 (1961). For example, cyanogen bromide cleaves peptides at methionine residues. Gross & Witkip, J. Am Chem Soc., 83: 1510 (1961). Thus, by treating full-length oncogene peptides with various combinations of modifiers, proteolytic enzymes and/or chemical reagents, numerous discrete overlapping peptides of varying sizes are generated. These peptide fragments

can be isolated and purified from such digests by chromatographic methods. Most preferably, isolated peptides of the present invention can be synthesized using an appropriate solid state synthetic procedure. Peptide Synthesis, Freeman, San Francisco, CA (1968). A peptide synthesis, Hormone Res., 23: 451 (1967). See also Recent Progress in Hormone Res., 23: 451 (1967). For example 2. The antigenic activity of these peptides may be tested using, for example, the assays as convenient. It may be purified by standard methods indicated herein.

Once an isolated peptide of the invention is obtained, chromatography (e.g., ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for protein purification. For immunoaffinity chromatography, a mutant HHR2/neu peptide may be isolated by binding it to an affinity column comprising antibodies that were raised against that peptide, or a related peptide of the invention, and were affixed to a stationary support.

Alternatively, affinity tags such as hexa-His (Invitrogen), maltose binding domain (New England Biolabs), influenza coat segment (Kodak), and glutathione-S-transferase can be attached by passage over an appropriate affinity column. A DNA affinity column using DNA containing a sequence encoding the peptides of the invention can be used in the purification of the peptides of the invention to allow easy purification of the peptides of the invention.

509, 1991), and glutathione-S-transferase can be attached to the peptides of the invention to allow easy purification by passage over an appropriate affinity column. A DNA sequence containing domain (New England Biolabs), influenza coat segment (Kodak) et al. Methods Enzymol. 194: 508-

20 Maltose binding domain (New England Biolabs), influenza coat segment (Kodak), and glutathione-S-transferase can be attached to the peptides of the invention to allow easy purification by passage over an appropriate affinity column. A DNA sequence containing domain (New England Biolabs), influenza coat segment (Kodak) et al. Methods Enzymol. 194: 508-

25 Maltose binding domain (New England Biolabs), influenza coat segment (Kodak), and glutathione-S-transferase can be attached to the peptides of the invention to allow easy purification by passage over an appropriate affinity column. A DNA sequence containing domain (New England Biolabs), influenza coat segment (Kodak) et al. Methods Enzymol. 194: 508-

30 Maltose binding domain (New England Biolabs), influenza coat segment (Kodak), and glutathione-S-transferase can be attached to the peptides of the invention to allow easy purification by passage over an appropriate affinity column. A DNA sequence containing domain (New England Biolabs), influenza coat segment (Kodak) et al. Methods Enzymol. 194: 508-

35 Maltose binding domain (New England Biolabs), influenza coat segment (Kodak), and glutathione-S-transferase can be attached to the peptides of the invention to allow easy purification by passage over an appropriate affinity column. A DNA sequence containing domain (New England Biolabs), influenza coat segment (Kodak) et al. Methods Enzymol. 194: 508-

cleavage, linkage to an antibody molecule, membrane or after translocation, e.g., by phosphotranslation, proteolytic antigenic peptides that are differentially modified during also included within the scope of the invention are magnetic resonance, and x-ray crystallography.

Also included within the scope of the invention are purification. Isolated peptides can also be physiologically active peptides of the invention can be used in the purification of the peptides of the invention.

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Also included within the scope of the invention are magnetic resonance, and x-ray crystallography.

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olecule or other ligand, (Ferguson, et al., Ann. Rev. Biochem. 57:285-320, 1988).

ISOLATED NUCLEIC ACID SEQUENCES

Another aspect of the invention are isolated nucleic acid sequences that encode the antigenic peptides described herein. With regard to nucleic acid sequences of the present invention, "isolated" means: an RNA or DNA polymer, portion of genomic nucleic acid, cDNA, or synthetic nucleic acid which, by virtue of its origin or manipulation: (i) is not associated with all of a nucleic acid with which it is associated in nature (e.g. is present in a host cell as a portion of an expression vector); or (ii) is linked to a nucleic acid or other chemical moiety other than that to which it is linked in nature; or (iii) does not occur in nature. By "isolated" it is further meant a nucleic acid sequence: (i) amplified in vitro by, for example, polymerase chain reaction (PCR); (ii) synthesized by, for example, chemical synthesis; (iii) recombinantly produced by cloning; or (iv) purified, by cleavage and gel separation.

The nucleic acid sequences of the present invention may be characterized, isolated, synthesized and purified using more than ordinary skill. See Sambrook et al., Molecular Cloning, Cold Spring Harbor Press, New York, 1989, incorporated herein by reference.

1. Functional equivalents

The term "functional equivalent", when applied to the nucleotide sequences of the invention, describes a sequence that satisfies one of the following conditions: (i) the nucleotide sequence in question can hybridize to a naturally-occurring mutant HER2/neu oncogene sequence, but does not necessarily hybridize to that oncogene sequence with an affinity that is the same as that of the naturally occurring, mutant HER2/neu oncogene sequence; (ii) the nucleotide sequence in question can serve as a probe to

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5 Due to the degeneracy of nucleotide coding sequences
 (see Alberes et al., Molecular Biology of the Cell, Garland Publishing, New York and London, 1989 - page 103),
 due to the degeneracy of nucleotide coding sequences
 note (i.e., mutant HRA-2 binds to different sequences and oncogenes and that do
 canonical HRA-2 binds to different sequences and those that do
 not (i.e., mutant HRA-2 binds to different sequences and oncogenes and that do
 oncogene sequences).

10 Incorporated herein by reference), a number of different
 nucleic acid sequences may be used in the practice of the
 present invention. These include, but are not limited to,
 sequences comprising SRQ ID NO.: 7 (encoding the mutant
 HRA-2/neu amino acid sequence of SRQ ID NO.: 2) and that
 have been altered by the substitution of different codons
 encoding the same amino acid residue within the sequence,
 thus producing a silent change. Almost every amino acid
 except tryptophan and methionine is represented by several
 different codons differing only in the third base. This
 feature, together with a tendency for similar amino acids
 to be represented by related codons, increases the
 probability that a single, random base change will result
 in no amino acid substitution or in one involving an amino
 acid of similar character.

15 For example, several different nucleotide sequences are
 capable of encoding the amino acid sequence of SRQ ID NO.: 2
 which is the mutant HRA-2/neu peptide, and SRQ ID
 NO.: 1, which is the wild type HRA-2/neu peptide. Nucleotide
 sequences capable of encoding the mutant HRA-2/neu peptide
 can be summarized as the sequence 5'-3', (SRQ ID NO.: 8-11),
 where Y represents C or T/U, H represents A, C or T/U, R
 represents A, G, and N represents A, C, G, or T/U. Such
 degenerate nucleotide sequences are regarded as functional
 equivalents of the specificially claimed equivalents. Nucleic
 acid sequences encoding the amino acid sequence of SRQ ID
 NO.: 3 are also included within the scope of the invention.

20 In no amino acid substitution or in one involving an amino
 acid of similar character.

25 For example, several different nucleotide sequences are
 capable of encoding the amino acid sequence of SRQ ID NO.: 2
 which is the mutant HRA-2/neu peptide, and SRQ ID
 NO.: 1, which is the wild type HRA-2/neu peptide, and SRQ ID
 NO.: 1, which is the mutant HRA-2/neu peptide, and SRQ ID
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 sequences capable of encoding the mutant HRA-2/neu peptide
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 represents A, G, and N represents A, C, G, or T/U. Such
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 acid sequences encoding the amino acid sequence of SRQ ID
 NO.: 3 are also included within the scope of the invention.

30 In no amino acid substitution or in one involving an amino
 acid of similar character.

35 For example, several different nucleotide sequences are
 capable of encoding the amino acid sequence of SRQ ID NO.: 2
 which is the mutant HRA-2/neu peptide, and SRQ ID
 NO.: 1, which is the wild type HRA-2/neu peptide, and SRQ ID
 NO.: 1, which is the mutant HRA-2/neu peptide, and SRQ ID
 NO.: 1, which is the wild type HRA-2/neu peptide. Nucleotide
 sequences capable of encoding the mutant HRA-2/neu peptide
 can be summarized as the sequence 5'-3', (SRQ ID NO.: 8-11),
 where Y represents C or T/U, H represents A, C or T/U, R
 represents A, G, and N represents A, C, G, or T/U. Such
 degenerate nucleotide sequences are regarded as functional
 equivalents of the specificially claimed equivalents. Nucleic
 acid sequences encoding the amino acid sequence of SRQ ID
 NO.: 3 are also included within the scope of the invention.

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The nucleotide sequences of the invention (e.g. SEQ ID NOS.: 7-11) can be altered by mutations such as substitutions, additions or deletions that provide for functionally equivalent nucleic acid sequence. In particular, a given nucleotide sequence can be mutated in vitro or in vivo, to create variations in coding regions and/or to form new restriction endonuclease sites or destroy preexisting ones and thereby to facilitate further in vitro modification. Any technique for mutagenesis known in the art can be used including, but not limited to, in vitro site-directed mutagenesis (Hutchinson, et al., J. Biol. Chem. 253:6551, 1978), use of TAB® linkers (Pharmacia), PCR-directed mutagenesis, and the like. The functional equivalence of such mutagenized sequences, as compared with un-mutagenized sequences, can be empirically determined by comparisons of structural and/or functional characteristics.

2. Isolated nucleic acid sequences of other oncogenes

The information provided on the HER2/neu mutant sequences described above allows the development of a general protocol for isolating any other oncogene nucleotide sequences encoding peptides that are capable of binding to the HLA-A2 cleft. In particular, nucleic acids encoding peptides containing the canonical sequences (e.g., SEQ ID NOS.: 3) can easily be identified and synthetic oligonucleotides produced using well-known methods.

Alternately, it could be valuable to perform PCR reactions using as a DNA template a nucleotide sequence known to contain at least one nucleotide sequence that encodes a functional equivalent of SEQ ID NO.2 or 3. By way of example only, SEQ ID NOS.:8-11 are degenerate oligonucleotides that may be used as PCR primers to isolate nucleotide sequences from other oncogenes that contain sequences encoding the canonical mutant peptide sequences described herein. For instance, PCR may be performed in 25

el ectroporation, etc. See Examples 3 and 7.

example, transformation, transfection, infection, for instance can be introduced into host cells by, for sequences that encode mutant HRS2/neu peptides of the recombinant vectors containing eukaryotic nucleotide 4. Introduction into and expression in host cells

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plasmid derivatives.

PBR, PUC, PGEM (Promega), and Bluescript (Stratagene) reference). Plasmids include, but are not limited to, total sequences 7:980-990, 1984, incorporated herein by as amphotropic murine retroviruses (see Miller and Rosman viruses. Viral vectors also include retroviral vectors, such bovine papillomaviruses, Rous-Sarcoma virus, and vaccinia include, but are not limited to, Lambda, simian viruses, compatible with the host cell used. Viral vectors or modified viruses, although the vector system must be vectors include, but are not limited to, cosmids, plasmids cloned into any of a large variety of vectors. Possible particular, nucleotide sequences of the invention may be constituents of which are incorporated herein by reference. In Cold Spring Harbor Press, New York, 1989), the entire (see, for example, Sambrook, J. et al., Molecular Cloning, be cloned or subcloned using any method known in the art. The isolated nucleotide sequences of the invention may be associated with vector sequences

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nucleotide sequence capable of encoding at least SEQ ID peptide of the invention includes clones of any eukaryotic of a nucleotide sequence that encodes a mutant antigenic 30 seconds at 55°C and 1 minute at 72°C. Preferred clones breast cancer tissue with 30 cycles of 30 seconds at 94°C, polymerase, 5 pm each primer and 0.4 ug human DNA from MgCl₂, 0.01% gelatin, 50 μM each dNTP, 1.5 unit Tag DNA 0.1 volumes with 10mM Tris buffer pH8.5, 50mM KCl, 3mM

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NO.2.

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Recombinant vectors containing nucleotide sequences encoding the mutant HER2/neu peptide of the invention can be engineered such that the eukaryotic nucleotide sequences are placed under the control of regulatory elements (e.g. 5 promoter sequences, polyadenylation signals, etc.) in the vector sequences. Such regulatory elements can function in a host cell to direct the expression and/or processing of nucleotide transcripts and/or peptide sequences of the invention.

10 Expression systems can utilize prokaryotic and/or eukaryotic (i.e., yeast, human) cells. See, for example, "Gene Expression Technology", Volume 185, Methods in Enzymology, (ed. D.V. Goeddel), Academic Press Inc., (1990) and U.S. Patent 5,229,115 "Adoptive Immunotherapy with

15 Interleukin-7" (Lynch: July 20, 1993) incorporated herein by reference. A large number of vectors have been constructed that contain powerful promoters that generate large amounts of mRNA complementary to cloned sequences of DNA introduced into the vector. For example, and not by

20 way of limitation, expression of eukaryotic nucleotide sequences in *E. coli* may be accomplished using lac, trp, lambda, and recA promoters. See, for example, "Expression in *Escherichia coli*", Section II, pp. 11-195, V. 185, Methods in Enzymology, supra; see also Hawley, D.K., and

25 McClure, W.R., "Compilation and Analysis of *Escherichia coli* promoter DNA sequences", Nucl. Acids Res., 11: 4891-4906 (1983), both of which are incorporated herein by reference. Expression of peptides of the invention in a recombinant bacterial expression system can be readily

30 accomplished..

Yeast cells suitable for expression of the mutant peptides of the invention include the many strains of *Saccharomyces cerevisiae* (see above) as well as *Pichia pastoris*. See, "Heterologous Gene Expression in Yeast", Section IV, pp. 231-482, V. 185, Methods in Enzymology, supra, incorporated herein by reference. Moreover, a large number of vector-mammalian host systems known in the

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art may be used. See, Sambrook et al., Volume III, *supra* and "Bxpressiion of Heterologous Genes in Mammalian Cells", Section V, pp. 485-596, V. 185, *Methods in Enzymology*, usually supply a promoter and other elements such as viral expression vectors derived from simian virus 40 (SV 40), retrovirus, and baculovirus. These vectors are transitory or stably express DNA and those that involve polycladenylation bigmals. Possible vectors include not limited to, cosmids, plasmids or modified viruses, but the vector system must be comparable with the host cell not limited to, cosmids, plasmids or modified viruses, but are used. Viral vectors include, but are not limited to, vaccinia virus, or lambda derivatives. Plasmids include, can be introduced into host cells via transfection, transfection, infection, electroporation, etc. Generally, host is accomplished using a vector containing DNA encoding the peptide under the control of regulatory regions that function in the host cell.

In particular, expression systems that provide for overproduction of a nucleotide sequence encoding the mutant HRR2/neu peptides of the invention can be prepared using for example, the methods described in U.S. Patent 4,820,642 (Kdman et al., April 11, 1989), incorporated herein by reference. The general requirements for preparing one form of expression vector capable of overexpression are: (1) the presence of a gene (e.g., a eukaryotic gene) into which a nucleotide sequence capable of encoding a mutant HRR2/neu peptide can be inserted; (2) the promoter of this eukaryotic gene; and (3) a second promoter located upstream from the eukaryotic gene which overexpresses the peptide can be inserted.

of expression vector capable of overexpression are: (1) the presence of a gene (e.g., a eukaryotic gene) into which a nucleotide sequence capable of encoding a mutant HRR2/neu peptide can be inserted; (2) the promoter of this eukaryotic gene; and (3) a second promoter located upstream from the eukaryotic gene which overexpresses the peptide can be inserted.

of expression vector capable of overexpression are: (1) the presence of a gene (e.g., a eukaryotic gene) into which a nucleotide sequence capable of encoding a mutant HRR2/neu peptide can be inserted; (2) the promoter of this eukaryotic gene; and (3) a second promoter located upstream from the eukaryotic gene which overexpresses the peptide can be inserted.

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obtained in any suitable manner. Possible host cells into which recombinant vectors containing nucleotide sequences can be introduced include, for example, bacterial cells, yeast cells, non-human mammalian cells in tissue culture or in situ, and human cells in tissue culture but not in situ.

Nucleotide sequences that have been introduced into host cells for encoding the mutant HER2/neu peptides of the invention can exist as extra-chromosomal sequences or can be integrated into the genome of the host cell by homologous recombination, viral integration, or other means. Standard techniques such as Northern blots and Western blots can be used to determine that introduced sequences are in fact being expressed in the host cells.

In one method of expressing a human HER2/neu peptide, a cDNA clone that contains SEQ ID NO.:2 is cloned into a expression vector and transfected into T2 cells (See also Example 7). Expression is monitored after transfection by, for example, Northern , Southern, or Western blotting.

C. ANTIBODIES

The term "antibodies" is meant to include monoclonal antibodies, polyclonal antibodies and antibodies prepared by recombinant nucleic acid techniques that are selectively reactive with peptides of the present invention. The term "selectively reactive" refers to those antibodies that react with one or more antigenic determinants of a peptide and do not react with other polypeptides. Antigenic determinants usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and have specific three dimensional structural characteristics as well as specific charge characteristics. Antibodies can be used for diagnostic applications or for research purposes.

In particular, antibodies may be raised against amino-terminal (N-terminal) or carboxy-terminal (C-terminal) residues of the mutant HER2/neu peptide of the invention.

Generally, to isolate antibodies to a mutant HbR2/neu peptide of the invention, peptide equivalents of its function, peptide SEQ ID NO.: 2 or its functional equivalent may be selected as an immunogen. This peptide immunogen can correspond to the mutant HbR2/neu peptide of the invention, certain amino acid sequences are more likely than others to provoke an immediate response, for example, than a prokaryotic (e.g., bacterial) or eukaryotic cell; the inclusion: (i) immunizing an animal with a protein expressed as a fusion protein in a host using standard techniques (see above); see Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, New York: 1989) such that 5-20% of the total peptide that can be recovered from the host are peptides HbR2/neu peptides of the invention. Recovered mutant HbR2/neu peptides of the invention, a mutant peptide sample can then be eluted from the gel, elute and prepared for immunization. Alternatively, a mutant HbR2/neu peptide can be eluted from the gel. The desirably cross-reactive with a mutant HbR2/neu peptide of the invention by using conventional methods such as, for example, ion exchange chromatography, size exclusion, or affinity chromatography. Once the mutant HbR2/neu peptide immunogen is prepared, mice can be immunized twice intraperitoneally with approximately 50 micrograms of peptide immunogen per mouse. sera from such immunized mice can be tested for antibody activity by immunoassay or immunochemistry on mouse.

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any host system expressing the mutant HER2/neu and by ELISA with the expressed mutant HER2/neu peptide. For immunohistology, active antibodies of the present invention can be identified using a biotin-conjugated anti-mouse immunoglobulin followed by avidin-peroxidase and a chromogenic peroxidase substrate. Preparations of such reagents are commercially available; for example, from Zymac Corp., San Francisco, California. Mice whose sera contain detectable active antibodies according to the invention can be sacrificed three days later and their spleens removed for fusion and hybridoma production. Positive supernatants of such hybridomas can be identified using the assays described above and by, for example, Western blot analysis.

To further improve the likelihood of producing an antibody as provided by the invention, the amino acid sequence of peptides of the present invention may be analyzed in order to identify portions of amino acid sequence which may be associated with increased immunogenicity. For example, peptide sequences of the invention may be subjected to computer analysis to identify potentially immunogenic surface epitopes. Such computer analysis can include generating plots of antigenic index, hydrophilicity, structural features such as amphophilic helices or amphophilic sheets and the like.

For preparation of monoclonal antibodies directed toward peptides of the invention, any technique that provides for the production of antibody molecules by continuous cell lines may be used. For example, the hybridoma technique originally developed by Kohler and Milstein (Nature, 256: 495-497, 1973), as well as the trioma technique, the human B-cell hybridoma technique (Kozbor et al., Immunology Today, 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies, and the like, are within the scope of the present invention. See also Akiyama et al. (Science, 232: 1644-1646, 1986, incorporated herein by reference) who teaches

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exhaustive of the various classes of coupling agents known in the art but, rather, is exemplary of the more common coupling agents. (See Killen and Lindstrom 1984, "Specific killing of lymphocytes that cause experimental Autoimmune Myesthenia Gravis by toxin-acetylcholine receptor conjugates." Jour. Immun. 133:1335-2549; Jansen, F.K., H.E. Blythman, D. Carriere, P. Casella, O. Gros, P. Gros, J.C. Laurent, F. Paolucci, B. Pau, P. Poncelet, G. Richer, H. Vidal, and G.A. Voisin. 1982. "Immunotoxins: Hybrid molecules combining high specificity and potent cytotoxicity". Immunological Reviews 62:185-216; and Vitetta et al., *supra*).

Preferred linkers are described in the literature. See, for example, Ramakrishnan, S. et al., Cancer Res. 44:201-208 (1984) describing use of MBS (M-maleimidobenzoyl-N-hydroxysuccinimide ester). See also, Umemoto et al. U.S. Patent 5,030,719, describing use of halogenated acetyl hydrazide derivative coupled to an antibody by way of an oligopeptide linker. Particularly preferred linkers include: (i) EDC (1-ethyl-3-(3-dimethylamino-propyl) carbodiimide hydrochloride; (ii) SMPT (4-succinimidylcarbonyl-alpha-methyl-alpha-(2-pyridyl-dithio)-toluene (Pierce Chem. Co., Cat. #21558G); (iii) SPDP (succinimidyl-6 [3-(2-pyridyl)dithio] propionamido] hexanoate (Pierce Chem. Co., Cat #21651G); (iv) Sulfo-LC-SPDP (sulfosuccinimidyl 6 [3-(2-pyridyl)dithio]-propianamide] hexanoate (Pierce Chem. Co. Cat. #2165-G); and (v) sulfo-NHS (N-hydroxysulfo-succinimide: Pierce Chem. Co., Cat. #24510) conjugated to EDC.

The linkers described above contain components that have different attributes, thus leading to conjugates with differing physio-chemical properties. For example, sulfo-NHS esters of alkyl carboxylates are more stable than sulfo-NHS esters of aromatic carboxylates. NHS-ester containing linkers are less soluble than sulfo-NHS esters. Further, the linker SMPT contains a sterically hindered disulfide bond, and can form conjugates with increased

D. DIAGNOSTIC APPLICATIONS

The known sequences of the peptides of the present invention now permit the screening of any cell sample for the expression of these peptides. For example, peptides may be extracted from the surface of cells and exposed in a Western blot to antibodies selective for the specific peptide of the invention. The presence of a positive Western blot to antibodies selective for the specific peptide of the invention indicates the presence of the peptide in the sample. The culture media may be extracted from tissues by, for instance, the quantitation method. See Current Protocols in Immunology, supra.

Peptide. In another technique, total RNA is isolated from peptide. In another technique, total RNA is separated from total RNA using a oligo (dt) column. The bound poly (A+) RNA is eluted by removing salt from the solution, thus destabilizing the poly (A+) RNA by hydrida. RNA is then transcribed into DNA using reverse transcriptase. The obtained DNA is amplified by PCR using two DNA primers. A set of PCR primers is selected, one primer hybridizing to the DNA sequences is selected, one primer hybridizing upstream of the mutation and one primer downstream of the HRR2/neu mutation and one HRR2/neu primer hybridizing upstream of the mutation. Thus, only the DNA segment overlapping the mutation will be amplified by PCR. Whether the cell sample expresses wild-type or mutated HRR2/neu. It is possible to select DNA primers such that HRR2/neu. The mutant DNA will be amplified, not the wild-type.

D. DIAGNOSTIC APPLICATIONS

than general, less stable linkages because the disulfide linkage is than other linkages because the disulfide linkage is cleaved in water, resulting in less conjugate available. Sulfon-NHS, in particular, can enhance the stability of carbodimide couplings. Carbodimide conjugations (such as RDC) when used in conjunction with sulfo-NHS, forms esters that are more resistant to hydrolysis than the carbodimide coupling reaction alone.

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E. IMMUNOTHERAPEUTIC APPLICATIONS

1. Compositions

The nucleotide sequences and polypeptides expressed by the sequences described herein can be used in pharmaceutical compositions in, for example, adoptive immunotherapy, peptide vaccine therapy and/or gene therapy. An exemplary pharmaceutical composition is a therapeutically effective amount of mutant HER2/neu nucleotide sequence of the invention or a mutant HER2/neu antigenic peptide optionally included in a pharmaceutically-acceptable and compatible carrier. The term "pharmaceutically-acceptable and compatible carrier" as used herein, and described more fully below, refers to (i) one or more compatible solid or liquid filler diluents or encapsulating substances that are suitable for administration to a human or other animal, and/or (ii) a system, such as a retroviral vector, capable of delivering, for instance, the mutant HER2/neu nucleotide sequence to a target cell. In the present invention, the term "carrier" thus denotes an organic or inorganic ingredient, natural or synthetic, with which the antigenic peptides and nucleic acid sequences of the invention are combined to facilitate application. Another exemplary carrier is, for example the T2 cell line (See Example 5) or other cells (e.g., fibroblasts) which may present on their surfaces the antigenic peptides of the invention.

The term "therapeutically-effective amount" is that amount of the present pharmaceutical compositions which produces a desired result or exerts a desired influence on the particular condition being treated. Various concentrations may be used in preparing compositions incorporating the same ingredient to provide for variations in the age of the patient to be treated, the severity of the condition, the duration of the treatment and the mode of administration.

The term "compatible", as used herein, means that the components of the pharmaceutical compositions are capable

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Dose of the present pharmaceutical compositions of the invention will vary depending on the subject and upon part of the route of administration used. By way of an example, an overall dose range of from about 1 to 300 micrograms to about 300 micrograms is a contemplated for human use. This dose can be delivered on at least two separate occasions, preferably spaced apart by about 1 week. See 5893 (1988), incorporated herein by reference.

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also be administered to a subject according to a variety of other, well-characterized protocols. For example, certain currently accepted immunization regimens can include the following: (i) a first dose at elected date; a second dose at 1 month after first dose; and a third dose at 5 months after second dose. See Product Information, Phytician's Desk Reference, Merck Sharp & Dohme (1990), at 1442-43.

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(e.g., Hepatitis B Vaccine-type protocol); (ii) Recommended administration for children is a first dose at elected date (at age 6 weeks old or older); a second dose at 4-8 weeks after first dose; a third dose at 6-12 months after third dose; a dose: a fourth dose at age 4-6 years old; and additional booster every 10 years after last dose. See Product Information, Phytician's Desk Reference, Merck Sharp & Dohme (1990), at 879 (e.g., Diphtheria, Tetanus and Pertussis-type vaccine).

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multiple doses of a particular composition can be administered by one of ordinary skill in the art employing more than routine experimentation.

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The peptides of the invention may also be administered peptide (peptidase) or in the form of a pharmaceutically acceptable salt. When used in medicine, the salts should be esterified to the peptide to form a pharmaceutically acceptable salt.

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be pharmaceutically acceptable, but non-pharmaceutically acceptable salts may conveniently be used to prepare pharmaceutically acceptable salts thereof and are not excluded from the scope of this invention. Such 5 pharmaceutically acceptable salts include, but are not limited to, those prepared from the following acids: hydrochloric, hydrobromic, sulphuric, nitric, phosphoric, maleic, acetic, salicylic, p-toluene-sulfonic, tartaric, citric, methanesulphonic, formic, malonic, succinic, 10 naphthalene-2-sulfonic, and benzenesulphonic. Also, pharmaceutically acceptable salts can be prepared as alkaline metal or alkaline earth salts, such as sodium, potassium or calcium salts of the carboxylic acid group. Thus, the present invention also provides pharmaceutical 15 compositions, for medical use, which comprise nucleic acids and/or peptides of the invention together with one or more pharmaceutically acceptable carriers thereof and optionally any other therapeutic ingredients.

The compositions include those suitable for oral, 20 rectal, topical, nasal, ophthalmic or parenteral administration, all of which may be used as routes of administration using the materials of the present invention. Other suitable routes of administration include 25 intrathecal administration directly into spinal fluid (CSF), direct injection onto an arterial surface and intraparenchymal injection directly into targeted areas of an organ. Compositions suitable for parenteral administration are preferred. The term "parenteral" includes subcutaneous injections, intravenous, 30 intramuscular, intrasternal injection or infusion techniques.

The compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. All methods include the step 35 of bringing the active ingredients of the invention into association with a carrier which constitutes one or more accessory ingredients.

5 Compositions of the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets, tablets or lozenges, each containing a predetermined amount of the nucleic acids and/or peptides aqueous liquid or non-aqueous liquid such as a syrup, an elixir, or an emulsion.

10 Preparation of the nucleic acids and/or peptides aqueous liquid composition conveniently comprise a sterile aqueous suspension suitable for parenteral injection according to known methods using those suitable dispersing agents. The sterile or wetting agents and suspending agents. The sterile solvent or suspension may also be a solvent that may be employed are water, Ringer's solution and isotonic sodium chloride solution. In addition, solvents that may be employed are 1,3-butane diol. Among the acceptable vehicles and acceptable diluent or solvent, for example as a solution in acetone or suspension medium. For this purpose any bland solvent or suspending medium. For this purpose any bland solvent or suspending medium. For this purpose any bland solvent or suspending medium. For this purpose any bland solvent or suspending medium. For this purpose any bland solvent or suspending medium.

15 The nucleic acids and/or peptides of the present and use in the preparation of injectables.

20 The nucleic acids are conjugated to a moiety for use in invention can also be conjugated to a moiety for use in vaccines. The moiety to which the nucleic acids and/or polypeptides is conjugated can be a protein, carbohydrate, lipid, and the like. See discussion of available linkers, the moiety to which nucleic acids and/or peptides may be intended to limit the scope of the invention in any way.

25 The nucleic acids and/or peptides of the present invention can also be included in any substance which incorporates the nucleic acids and/or peptides of the present invention.

30 The chemical structure of this moiety is not

35 and/or peptides of the invention which potentiates the into or administered simultaneously with the nucleic acids intended to include any substance which is incorporated bound can also be an adjuvant. The term "adjuvant" is the moiety to which nucleic acids and/or peptides may be intended to limit the scope of the invention in any way.

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immune response in the subject. Adjuvants include aluminum compounds, e.g., gels, aluminum hydroxide and aluminum phosphate gels, and Freund's complete or incomplete adjuvant. The paraffin oil may be replaced with different types of oils, e.g., squalene or peanut oil. Other materials with adjuvant properties include BCG (attenuated Mycobacterium tuberculosis plus other microbial derivatives), calcium phosphate, levamisole, isoprinosine, polyanions (e.g., poly A:U), leutinan, pertussis toxin, lipid A, saponins and peptides, e.g., muramyl dipeptide. Rare earth salts, e.g., of lanthanum and cerium, may also be used as adjuvants. The amount of adjuvant required depends upon the subject and the particular therapeutic used and can be readily determined by one skilled in the art without undue experimentation.

An exemplary composition of the invention can comprise a therapeutically effective amount of the isolated mutant peptide of the invention, either alone or in combination with an immunoadjuvant and/or carrier.

2. Methods of sensitizing lymphocytes *in vitro*

Current adoptive cancer immunotherapy procedures involve isolating tumor-infiltrating lymphocytes (TIL) from fresh surgical specimens of solid tumors, growing these TIL's to therapeutic numbers (10^{10} - 10^{11}), and reinfusing these cells back into the patients. The intention is that these T-lymphocytes will demonstrate anti-tumor activity due to their prior *in vivo* stimulation and selection within the environment of the tumor from which they came. The term "isolated", when applied to a population of T-lymphocytes means T-lymphocytes separated from naturally-occurring biological materials (See Examples 3 and 6) or associated with chemical moieties (e.g. peptides, carbohydrates, fatty acids, and the like) other than those associated with the T-lymphocytes in their naturally-occurring state.

Unfortunately, this *in vivo* activation is inefficient or the tumor would not exist. We have shown that the anti-

reference. See also Example 6. The resulting peptide
al., J. Immunol. 147:4047, incorporated herein by
with the peptide repeatedly as described by Bedmar et
ID NO. 2 alone initially, then with autoalogous PBL pulsed
phlebotomy. The PBL are cultured, for instance, with SBO
which are easily obtained from patients by simple
lymphocytes (CTL) from peripheral blood lymphocytes (PBL)
makes it feasible to generate tumor-specific cytotoxic T
isolation of the antigenic peptide of the invention
invasive procedures to obtain ascites fluid. The
must undergo surgical resection of the tumor or other
recognition; however, to acquire these cells, the patient
population of T-lymphocytes are based *in vivo* toward tumor
we currently use TAL's and TIL's because this
See Example 8.

associated lymphocytes (TAL) derived from ascites fluid.
peptides of the invention is also used to stimulate tumor
as with autoalogous tumor. T2 loaded with the antigenic
useful to stimulate TIL *in vitro* by repetitive stimulation
invention. The loaded T2 is very antigenic and, therefore,
competitively displaced by the antigenic peptides of this
presenting *in vitro* effect. These relative few peptides may be
relatively few different peptides, because of an antigen
other cell line capable of presenting the peptide). These
cells have the property that they express HLA-A2 bound with
be loaded in large quantities onto the T2 cell line or
by these tumor cells. Examples 4 and 5. The peptide may
autoalogous tumor, this antigenic peptide must be expressed
recognition by TIL which have been stimulated with
since we have demonstrated that SBO ID NO.: 2 is
because of the weak antigenicity of most tumors.
tumor, yet again this method is relatively ineffective
preferential expansion of TIL's with specificity for the
reference. This *in vitro* stimulation causes the
people et al. Surgery, 114:227 1993, incorporated herein by
repeatedly stimulating the TIL with autoalogous tumor. See
tumor activity of TIL can be enhanced *in vitro* by

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specific CTL's recognize the tumor efficiently when adoptively transferred in therapeutic numbers.

Potentially, fewer cells would be required to elicit a clinical response making the therapy more timely, less cumbersome, and less costly.

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3. Methods of sensitizing lymphocytes *in vivo*

Tumor vaccines are intended to stimulate the host immune system *in vivo* against the tumor of concern. These vaccines can be used as a therapeutic tool or preventative one. Currently, the majority of tumor vaccines are genetically-modified tumor cells designed to augment the host immune response against the tumor by either creating a more conducive tumor environment (i.e., tumor cells transfected with cytokine genes; See Golumbek, P. et al., Science 254: 713-716, 1991, incorporated herein by reference) or making the tumor more antigenic (i.e., transfecting tumors with the costimulant, B7; See, Townsend and Allison, Science 259: 368-370, 1993, incorporated herein by reference.).

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There are clinical trials already underway to test these vaccines in patients with end-stage disease (Rosenberg, NCI, melanoma; Pardol, Johns Hopkins, renal cell carcinoma; Chang, Michigan, melanoma). Under Dr. Rosenberg's protocol, genetically modified tumor cells are injected into the patients' thigh and an immune response allowed to occur. The draining inguinal lymph nodes are then harvested (and the injection site resected) and these *in vivo* - sensitized lymphocytes cultured and used for adoptive transfer back into the patient.

Cells such as T2 (or carrier cells such as autologous fibroblasts or B cell lines) are pulsed with an antigenic peptide of the invention (See Example 5) and similarly injected to produce a local immune reaction. The draining lymph nodes are harvested as a source of peptide/tumor-specific CTL for adoptive transfer. With the peptide/T2 (or fibroblast or B cell line), as opposed to the tumor

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supra for the influence virus matrix peptide. Other
sequence for the peptide as described by Bednarek et al.,
carries cell with episomal vectors containing the short DNA
autologous fibroblast, or B cell lines is to transfect the
Another way to insure peptide expression on the T2,
internalization of the complex.

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(such as with paraformaldehyde treatment) to prevent the
The peptide/HIA complex is fixed on the cell surface
cell lines which themselves pose no risk to the patient.
on a carrier cell like T2, autologous fibroblasts, or B
The vaccine is delivered as mentioned above or loaded
emulsifier.

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wall skeletons of mycobacterium phlet, squalene and
(monophosphoryl lipid A) from Salmoella minnesota, cell
Inc., Hamiltion, MT) contain detoxified endotoxin.
herein by reference). This adjuvant (Ribi Immunochem Res.
al., however, have demonstrated an effective protocol using
peptide would be degraded and not present. Much of the
host CTL's. This method may be ineffective as much of the
presenting cells (APC) which, in turn interact with the
beads) is delivered systematically and taken up by antigen-
ID NO.: 2 in combination with a synthetic carrier such as
primary cancers. A peptide vaccine (e.g., containing S9Q
those with predicted high risk factors for developing
cancer) are a first obvious group to vaccine, followed by
at high risk for recurrence (e.g., node-positive breast
cancers. Patients with previously treated malignancies but
promote active cellular protective immunity against certain
A peptide-based tumor vaccine may be designed to
procedure once again to the benefit of the patient.
the choice of infection sites and simplest is the harvest
lines pose no malignant risk to the patient. This broadens
not have been resected since the T2, fibroblast, or B cell
viable tumor cells. Furthermore, the injection site would
danger than injecting genetically modified, but still
cells, the patient would be exposed to much less potential

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cells, the patient would be exposed to much less potential

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vectors have been also used including poxvirus vector (Paoletti et al., "Immunotherapeutic Strategies for Cancer Using Poxvirus Vectors," Ann. NY Acad. Sci.; 292-300 incorporated herein by reference) and vaccinia vector (Bernards et al., Proc. Natl. Acad. Sci. 84:6854; incorporated herein by reference).

A tumor vaccine consisting of a nonthreatening cell expressing high amounts of a peptide of the invention would elicit a protective cellular response. The patient is then protected against future assaults by tumors expressing the same peptide.

We have shown that HER2/neu mutant peptide is expressed and recognized by breast, ovarian cancer, and lung cancer TIL's. Together, these tumors account for 65% of all female malignancies. The fact that the HER2/neu mutation also exists in gastric cancer (Yamamoto et al., Nature 319:230) opens the possibility that the mutated peptide exists in other gastrointestinal malignancies known to express HER2/neu such as pancreatic and colon cancer. Furthermore, since the mutated HER2/neu peptide is not found in normal tissue, which expresses wild-type HER2/neu (Coussens et al., Science 230:1132: see also SEQ ID NO.: 1), no destruction of normal tissue would be induced. Therefore, antigenic mutant peptides in general, and the mutant HER2/neu mutant peptides in particular, could form the basis of widely recognized, tumor-specific vaccines.

The invention will now be illustrated with the following non-limiting examples.

Example 1 - HLA-A2 Binding Assay

This Example illustrates a procedure adapted from Ruppert et al. (supra), for purifying HLA-A2 and performing a quantitative binding assay.

A2.1 Purification

The HLA-A2.1-positive human Epstein-Barr virus-transformed B cell line JY is used as a source of A2

10) cell cultures are filtered through 0.45 μm filters and purified by affinity chromatography. Columns of 10¹⁰ molecules. In brief, cell lysates from large-scale (10¹⁰) intact activated sepharose C14B and protein A-sepharose are used as precolumns. The cell lysate is subsequently depleted of HLA-B and HLA-C molecules by repeated passage over protein A-sepharose beads conjugated with anti-HLA-B/C antibody to remove HLA-B and HLA-C molecules. Subsequently, the anti-HLA-A/B/C antibody M6/32 is used to capture HLA-A2.1 molecules. Molecules are eluted with diethyldiamine, 1% a-ocyl glucoside (pH 11.5), neutralized with 1 M Tris (pH 6.8), concentrated by ultrafiltration on Amicon 30 (pH 6.8), and stored at 4°C. Protein purity, carboxylic acids, and effectiveness of depletion steps are monitored by SDS-polyacrylamide gel electrophoresis.

20 A quantitative assay for A2.1-binding peptides based on the inactivation of biotinylated radiolabeled standard peptides to detect biotinylation of biotinylated HLA molecules is described. In brief, a standard peptide (S9G ID NO.: 12; et al., 1987, incorporated herein by reference). HLA concentrations yielding approximately 15% of bound peptide are used in the inactivation assays (usually in the 10 nM range). Various doses of the test antigenic peptides of the inventors (ranging from 100 μM to 1 μM) are coincubated together with the ~ 5 nM radiolabeled standard peptide and 22 molecules, for 2 days at room temperature in the microglobulin. The final concentrations of peptide inhibitors are 1 μM pheylmethyleuropyl fluoride, 1.3 μM N-a-p-tosyl-L-lysine chloromethyl ketone. Final 1.10 phenanthroline, 73 μM pepstatin A, 8 μM EDTA, and 200 30 MHC-biotin are added to the reaction mixture. After 40. At the end of the incubation period, the percent of detergents concentration in the mixture is 0.05% nondet P-42.

35 MHC-biotin radiotoxicity is determined by gel filtration, 35 and the end of the incubation period, the percent of detergents concentration in the mixture is 0.05% nondet P-42.

25 MHC-biotin radiotoxicity is determined by gel filtration, 25 and the end of the incubation period, the percent of detergents concentration in the mixture is 0.05% nondet P-42.

20 MHC-biotin radiotoxicity is determined by gel filtration, 20 and the end of the incubation period, the percent of detergents concentration in the mixture is 0.05% nondet P-42.

15 MHC-biotin radiotoxicity is determined by gel filtration, 15 and the end of the incubation period, the percent of detergents concentration in the mixture is 0.05% nondet P-42.

5 MHC-biotin radiotoxicity is determined by gel filtration, 5 and the end of the incubation period, the percent of detergents concentration in the mixture is 0.05% nondet P-42.

10 MHC-biotin radiotoxicity is determined by gel filtration, 10 and the end of the incubation period, the percent of detergents concentration in the mixture is 0.05% nondet P-42.

1) cell cultures are filtered through 0.45 μm filters and purified by affinity chromatography. Columns of 10¹⁰ molecules. In brief, cell lysates from large-scale (10¹⁰) intact activated sepharose C14B and protein A-sepharose are used as precolumns. The cell lysate is subsequently depleted of HLA-B and HLA-C molecules by repeated passage over protein A-sepharose beads conjugated with anti-HLA-B/C antibody to remove HLA-B and HLA-C molecules. Subsequently, the anti-HLA-A/B/C antibody M6/32 is used to capture HLA-A2.1 molecules. Molecules are eluted with diethyldiamine, 1% a-ocyl glucoside (pH 11.5), neutralized with 1 M Tris (pH 6.8), and stored at 4°C. Protein purity, carboxylic acids, and effectiveness of depletion steps are monitored by SDS-polyacrylamide gel electrophoresis.

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and the 50% inhibitory dose calculated for each peptide, as described in Sette et al., 1992, J. Immunol. 148: 844-851, incorporated herein by reference.

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Example 2 - Peptide synthesis

Peptides are synthesized in the Biopolymer Laboratory at Brigham and Women's Hospital using solid phase techniques on an Applied Biosystems peptide analyser.

10 Syntheses are done by sequential coupling of N-a-Fmoc-protected amino acids on an Applied Biosystems 430A peptide synthesizer (Foster City, CA) using standard t-butoxycarbonyl- and 9-fluoromethyloxycarbonyl-protected amino acids and resins. Resins are treated with acetic
15 anhydride to block free, unreacted amino groups. Deprotection and removal of peptide from resins are performed as recommended by Applied Biosystems (430A Peptide Synthesizer Manual).

20 Crude products are dissolved in either water-acetic acid or DMSO-acetic acid mixtures. Solutes are filtered and injected onto C-18 semipreparative reverse phase HPLC columns (Waters Corp., Milford, MA) and eluted isocratically with TFA-acetonitrile gradients.

25 Identity and purity of the final products is established by amino acid analysis (Current Protocols in Immunology, *supra*) and mass spectrometry (Covey et al., Anal. Chem., 63: 1193, 1991). Purification is continued until single peaks were obtained by analytical HPLC (Current Protocols in Immunology, *supra*).

30 SEQ ID No.:2 was made about 40% pure by weight and it is extremely unlikely that a 9 amino acid peptide with a closely related but different sequence could be contaminating this peptide (personal communication, David Teplow, Director of Brigham and Women's Biopolymer Lab).
35 Because SEQ ID NO.: 2 is so hydrophobic, we experienced difficulties in dissolving it. We tried acetic acid, acetonitrile, and methanol all of which helped in

standard chromium release assays primarily because of the effect of pH on the cells. Dissolutions and mixtures of these solvents were also attempted. Finally, the peptides were dissolved as much as possible in water or HBS with prolonged time (as long as 36 hrs) and vortexing.

Example 3. Transfection of melanoma cell lines with the HHR2/neu gene.

This example illustrates the first observation in a human tumor system that HHR2/neu oncogene expression is related to induction of antigenicity.

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Laboratories) for 2 to 4 hours with continual stirring. Ascites fluid was collected in heparinized containers in the operation room, diluted 1:1 with HBSS without calcium or magnesium (Gibco Laboratories), and centrifuged at 200 g for 20 minutes to pellet the cells. Some ascites were centrifuged undiluted to freeze aliquots of steriley filtered ascites for tumor cultures. Red blood cells and cell debris were removed from the ascites cell pellet by centrifugation over 100% Ficoll (Organon Teknika Corporation, Durham, N.C.) for 20 minutes. Finally, both single cell suspensions (from ascites and solid tumor) were washed twice and lymphocytes and tumor cells were separated by centrifugation on discontinuous 75%/100% Ficoll gradients. The lymphocytes were collected at the lower interface and tumor cells were collected at the upper interface. Both populations were washed twice with HBSS, checked for viability, and counted with trypan blue (Gibco Laboratories) exclusion. Aliquots of TAL, TIL, ascitic tumor, and solid tumor were frozen in 90% fetal calf serum (FCS) (Gibco Laboratories) and 10% dimethyl sulfoxide (Sigma Chemical Company) at -80° C or used fresh.

Generation of tumor-specific CTL. TAL and TIL were suspended in complete lymphocyte medium composed of RPMI-1640 medium (Gibco Laboratories) with 20% Ventrrex (Ventrrex laboratories, Portland, Maine) and 10% FCS and supplemented with 50 units/ml penicillin and 50 µg/ml streptomycin (Gibco laboratories), 25 mmol/L HEPES buffer, and 2 mmol/L L-glutamine (Gibco Laboratories). Cultures were suspended at 5×10^5 cells/ml on solid phase anti-CD3 monoclonal antibody polystyrene plates (Orthoclone OKT3; Ortho Pharmaceutical Corporation, Raritan, N.J.) and placed in a humidified incubator at 37° C in 5% CO₂. After 48 hours T cells were transferred to uncoated 25 cm² flasks (Costar, Cambridge, Mass.) and supplemented with 50 IU/ml IL-2 (AMGEN, Thousand Oaks, Calif.). IL-2 was added at 50 IU/ml every 3 days. Cells were washed, counted and resuspended in fresh complete lymphocyte medium at 5×10^5 cells/ml.

chromate-51 (New England Nuclear, Boston, Mass.) for 1 hour the study. Targets were labeled with 50 to 100 μ Ci sodium alliogeneic ovarian targets came from the other patients in

fresh cryopreserved or early passage cultured tumor and the NK-sensitive K562 cell line were used as targets.

fresh cryopreserved or early passage cultured tumor and the standard 4-hour chromium (Cr)-release assays. Briefly,

cytotoxicity assays. Cytotoxicity was determined by

cytometer (Coulter Electromic, Hialeah, Fla.).

were used for two color analysis on a Coulter Epics

cell, CD8, CD16, and CD56 (Becton Dickinson, Mountain View, CD4, CD8, CD16, and CD56 (Becton Dickinson, Mountain View,

C. Monoclonal antibodies to CD3, T-cell receptor (TCR),

phycoerythrin-conjugated mAb (20 μ l) for 30 minutes at 40

$\times 10^6$ cells were doubly labeled with a fluororescent- and a

sampled periodically for changes in T-cell phenotype. Five

phenotype analysis. TAL and TIL bulk cultures were

cytotoxicity assays.

or used fresh when available as feeders and targets in

early passage cultured tumor cells were frozen in aliquots

(laboratories) with D-vialine substituted for D-vialine.

contrived with minimum essential fetal medium (Gibco

contaminant and expanded. In some cases, fibroblasts were

ethylenediaminetetraacetic acid; Gibco Laboratories) when

weakly. Cultures were trypsinized (0.05% trypsin and 0.02%

insulin was added every 3 days and the medium was changed

tumor, 10% by volume of sterilized filter sterilized fluid.

suspended at 1 \times 10 6 cells/ml and were supplemented with

antibody in RPMI-1640 medium plus 20% FCS. Cells were

and solid tumor cells were cultured in 25 cm 2 flasks

generation of tumor cells. Ascitic tumor cells

subfoxtail for future evaluation.

weekly aliquots were frozen in 90% FCS and 10% dimethyl

lymphocytes were maintained in culture up to 12 weeks.

cultures and solid tumor was used to feed TIL cultures.

lymphocyte: tumor ratio. Ascitic tumor was used to feed TAL

(10,000 rads) tumor cells were added at a 10:1

every week. At weeks 1, 3, and 5, autologous irradiated

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in a 37°C water bath with frequent resuspension of cells. Effectors were plated in 96-well round bottom plates (Costar) at designated effector:target ratios (usually 80:1 to 10:1) in 100 µl/well. The plates were centrifuged at 80 g for 5 minutes and placed in a humidified incubator at 37°C and 5% CO₂ for 4 hours. Culture supernatant was collected using a supernatant collection system (Skatron, Inc., Sterling, Va.) and radionuclide release was measured on a gamma counter (Gamma Tnc 1191; TM Analytic, Elk Grove, Ill.). All determinants were done in triplicate. Results are expressed as percentage specific lysis as determined by the equation % Lysis = [Experimental mean cpm - Spontaneous mean cpm] / [Maximum mean cpm - Spontaneous mean cpm] X 100 or lytic units, defined as the number of effector cells needed to lyse 20% of the targets and expressed per 10⁷ cells (LU₂₀/10⁷ cells).

Blocking of CTL activity by mAb

Target cells were incubated with 1/10-diluted supernatant of W6/32-producing hybridoma (anti-HLA class 1), L227-producing hybridoma (anti-HLA class 2) or BB7.2-producing hybridoma for 30 min at 4°C after labeling with chromium. The mAb were added to the effector cells and target cells in 1/4-dilution, and a 4 h-⁵¹Cr-release assay was performed as described above.

In situ hybridization

A 1.6 kb human neu cDNA (hu-neu, Oncogene Science) and a control DNA (pBR322, Oncogene Science) were labeled with biotin using a BioNick™ Labeling Kit (Gibco BRL) according to the instruction manual. Tumor cells (5 to 10 x 10⁴/100 ml CM) were put on a poly-L-lysine-coated slide and incubated at 37°C for 60 to 90 min. After washing with PBS, the plate was treated with 40 µg/ml of prewarmed proteinase K (Sigma) at room temperature (RT) for 2 min. The plate was serially treated with 4% paraformaldehyde for 1 min, 3 x PBS for 3 min, 1 x PBS for 6 min, and dehydrated through a graded ethanol series (50%, 70%, 90% and 100%)

for 3 min each. Hybridization of the labeled probe to the tissue slide was performed by using the *in situ* hybridization and detection system (Gibco BRL) according to the instruction manual. Brieftly, 200 ng/ml hybridization solution at RT for 15 min followed by addition of 100 μ l of streptavidin-alkaline phosphatase conjugate. After washing with tris-bufered saline (pH 7.5) for 30 min and with alkaline substrate buffer (100 mM Tris base, 150 mM NaCl, 50 mM MgCl₂, pH 9.5) for 5 min, the slide was washed with H₂O, dehydrated, mounted and observed with photomicroscopy.

Transfection of melanoma cells with the HHR2/neu gene. Transfection was performed by a lipofection method (Pegner et al., Proc. Nat. Acad. Sci., USA, 84: 7413, 1987) as described in the manufacturer's instructions (Gibco, BRL). Briefly, 1x10⁶ exponentially growing melanoma cells of two cell lines (A-Mn and G-Mn) were cultured in a 30-mm dish for 18 h in DMEM supplemented with 10% FCS. Five μ g of the plasmid containing the HHR2/neu gene (Yamamoto et al., Nature 319: 230, 1986; incorporated herein by reference) and the SV40 promoter and 1 μ g of pMNEO (Colbere-Garapin et al., J. Mol. Biol. 150: 1, 1981) containing the neomycin phosphotransferase gene and the dioloyl phosphatidyl-ethanolamine, and incubated at room temperature. This mixture was added to the melanoma cell culture, and aliquoted for 24 h at 37°C. After incubation, a liposome formulation of the cationic lipid N-(2,3-dihydroxypropyl)propyl)-N, N-trimethyl-aminomethyl chloride and moloney murine vector were combined with 15 μ g of lipofectin, a liposome formulation of the cationic lipid N-(2,3-dihydroxypropyl)propyl)-N, N-trimethyl-aminomethyl chloride and culture, and resuspended in fresh culture medium. Cells were washed, and resuspended for 24 h at 37°C. After incubation, culture, and aliquoted for 24 h at 37°C. After incubation, cells were washed, and resuspended in fresh culture medium.

Forty eight hours later, media was changed and 400 μ g/ml of culture, and aliquoted for 24 h at 37°C. After incubation, cells were washed, and resuspended in fresh culture medium.

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G418 was added for selection of transfectants (A-MM-neu/neo and G-MM-neu/neo). Controls were transfected with pMoNeo only (A-MM-neo and G-MM-neo). Transfection efficiency was 5X10⁴ to 2x10⁴.

5 Transfection was confirmed by flow cytometric assay of cell surface HER2/neu expression in the transfectants. A-MM-neu/neo and G-MM-neu/neo has 76% and 75.9% positive, respectively, and HLA class 1 expression was unaltered (data not shown). Flow cytometric analysis using the anti-
10 HLA-A2 monoclonal antibody BB7.2 revealed that A-MM is HLA-A2 positive and G-MM is HLA-A2 negative.

15 Six ovarian-specific CTL lines from six different patients were obtained; three of them (CTL 1,2 and 3) being HLA-A2 positive and CTL 4,5 and 6 being HLA-A2 negative. CTL 1,2 and 3 lysed A-MM-neu-neo transfectants (17-20% cytolysis) but neither G-MM-neu-neo transfectants (2-5% cytolysis) nor control transfectants (A-MM-neo and G-MM-neo: 2-5% cytolysis). CTL4 and 6 showed no cytotoxicity (2-5% cytolysis) against any of the transfectants. CTL 5
20 showed cytotoxicity against the A-MM transfectant (15% cytolysis) and its control (15% cytolysis), indicating that this cytotoxicity was not related to HER2/neu expression.

25 Sensitivity of the A-MM-neu-neo HLA-A2 positive transfectant was inhibited by anti HLA-A2 monoclonal antibody, but not by control antibody (data not shown).

30 Example 4. A Common Antigen System Exists between ovarian and breast cancers.

35 To determine if a common antigen system might exist between ovarian and breast cancers, we isolated tumor-infiltrating lymphocytes (TIL) from a series of HLA-A2⁺, HER2/neu⁺ ovarian (Ov) and breast (Br) cancer specimens. See Example 3. The TIL were cultured with repeated stimulation by irradiated autologous tumor cells until the cultures revealed tumor-specific cytotoxicity when tested against a series of HLA-unmatched allogeneic tumor targets in standard chromium-release assays at an E:T ratio of

Example 5. Recognition of mutant H-RB/neu peptide by cancer-specific lymphocytes. The TZ cell is a human T cell/B cell fusion product which has an antigen processing defect such that HLA-A2

Neither the OVT1 or the BT21 recognized HLA-A2⁺ melanomas, nor did HLA-A2⁻ OVT1 or BT21 lysate the HLA-A2⁺ cancer lines. The HBB2/neu expression in the HLA-A2⁺ and HLA-A2⁻ tumors was comparable. This data suggests that HLA-A2 serves as a restriction element in breast cancer also, but more importantly, at least one common tumor antigen system exists between these closely related malignancies and is presented by the HLA-A2 allele.

Similarly, the tumors were cultured, and their HR2/neu expression measured by immunofluorescence, and the expression level determined by positives used. HR2/neu expression was determined by immunofluorescence staining with TA-1 anti-HR2/neu antibodies of fluorescein isothiocyanate conjugated. The fluorescence was measured by flow cytometry.

20:1, as previously described in Example 3. The tumors were cultured in specific CII, lines were typed, and the HLA-A2+ cultures were utilized. HLA-A2 status was confirmed by immunofluorescence staining using B7.2 and MA2.1 anti-HLA-A2 monoclonal antibodies (ATCC, Rockville, MD). HLA-A2 negative tumor lines were the gift of the Gynecology Oncology Laboratory at Brigham & Women's Hospital and HLA-A2 negative breast cancer lines were obtained from the ATCC (ATCC # 829 and 863). All tumor lines were expressed comparable to HLA-A2 positive lines. All tumor lines were obtained from the ATCC (ATCC # 829 and 863).

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5 molecules are present on the cell surface with relatively few endogenous peptides. See Salter et al., Science, 255: 1264 (1992). T2 is unique in that it is known to express some empty HLA-A2 molecules but these are only transiently expressed on the cell surface since it is the peptide that stabilizes the HLA molecule. If the temperature is lowered the cell membrane turns over much slower. Therefore, we
10 performed a series of experiments altering the concentration of peptide used, length of loading time, and incubation temperature. The temperature and length of incubation was limited by the viability of the T2 which had already been exposed to an hour of chromium loading. The best loading occurred at 4°C for 2 hours; however, the cell viability dropped significantly. We currently perform the
15 assays in serum-free media which contains less possible proteins/peptides capable of interfering with the loading of T2. We utilize only the most healthy T2 which has been resuspended for 36 hours prior to the assay and load with 100-200 µg/ml of peptide dissolved in water for 1 hour at
20 37°C.

25 Non-small cell lung cancer TIL, OvTIL and BrTIL were tested in standard chromium release assays against the HLA-A2⁺, antigen-processing cell line, T2, either unloaded or pulsed with SEQ ID NOS.:4 or No.:2 peptide at an E:T ratio of 20:1. After being labelled with chromium, the T2 was incubated with 50-100 µg/ml of either peptide for 1 hr at 37°C prior to cytotoxicity assays.

30 Both OvTIL and BrTIL were also tested in standard chromium release assays against T2 pulsed with increasing concentrations of SEQ ID NO.: 2 peptide. All incubations were for 1 hr at 37°C, and the E:T ratio was constant at 20:1. Peak recognition occurred at 100 µg/ml. In another experiment, after T2 had been labelled with chromium and pulsed with SEQ ID NO.:2, anti-HLA-A2 mAb, BB7.2 or anti-
35 HLA-A,-B,-C mAb, W6/32 was added at 1:2.

After the T2 cell line was loaded with the synthetic peptides, and four HLA-A2⁺ ovarian cancer-specific TIL

lines recognized the SRQ ID NO.: 2 peptide but not the SRQ ID NO.: 4 peptide or the unloaded T2 was approximately 50% recognition of the peptide-pulsed T2 was approximately 100% for an HLA-A2⁺ breast cancer cell line. The difference in these CTL lines may be related to the inefficiency of our current readout system. SRQ ID NO.: 2 is extremely hydrophobic as it comes from the transmembrane portion of the protein, and this property made its production and use difficult. Of extreme interest, three HLA-A2⁺ breast cancer cells also recognized SRQ ID NO.: 2 peptide but not the SRQ ID NO.: 1 peptide. The level of killing was generally comparable to that found by the OVTIL, and ranged between 10-25% at an effector:target ratio of 20:1. This range could be related to the effectiveness and overall cytotoxic potential of the effectors utilized, but more likely the degree of recognition is secondary to the level of HIR2/neu expression in the autologous tumor with which these lines were stimulated.

An HLA-A2⁺ lung cancer-specific T-lymphocyte line also recognized SRQ ID NO.: 2 ("GP2") but not the SRQ ID NO.: 1 peptide ("GP1") or the unloaded T2 (Fig. 7). The level of killing was comparable to that found by OVTIL and BRTIL. See also Yoshimura et al., Cancer Res., 54: 3387-3390 (July 1994) who confirmed that SRQ ID NO.: 2 is a tumor antigen presented by HLA-A2 and recognized by non-small cell lung cancer-specific CTL.

Fig. 5 demonstrates that both OVTIL and BRTIL recognize SRQ ID NO.: 2 peptide ("GP2") in a dose-response manner with maximum lysis occurring at approximately 100 ug/ml. No significant recognition of SRQ ID NO.: 4 ("GP1") was found at any of these concentrations. The lysate of SRQ ID NO.: 2-pulsed T2 was confirmed as being HLA-A2-restricted by pulsed T2 was inhibited by anti-HLA-A2 monoclonal antibody (mab) blocking studies. The anti-HLA-A2 mab, BB7.2 completely inhibited the recognition of SRQ ID NO.: 2-pulsed T2 by non-small cell lung cancer-specific CTL.

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5 ID NO.:2 by both OvTIL and BrTIL (Fig. 6). The level of BB7.2 blocking was equal to that of the anti-HLA-A-B-C mAb, W6/32, demonstrating that HLA-A2 is the only class I molecule presenting this peptide, as expected. These findings suggest that both ovarian cancer-specific and breast cancer-specific CTL recognize a HER2/neu-derived peptide found only in cancerous cells.

10 Example 6: Generation of CTL Cultures Responsive to HER2/neu Peptide from Peripheral Blood Lymphocytes (PBL)

15 CTL cultures specific for SEQ ID No.:2 are generated as follows: Briefly, PBL's from HLA-A2⁺ donors are separated on Hypaque-Ficoll and incubated in RPMI 1640 plus 10% pooled human plasma at 10⁶/ml in the presence of 5 µg/ml antigenic HER2/neu peptide. After 3 days interleukin-2 (Amgen, Thousands Oaks, CA) is added (2 U/ml). Seven days later, cells are centrifuged (800 x g for 10 min) and resuspended at 5 x 10⁵ viable cells/ml in the presence of 5 x 10³/ml peptide-pulsed PBL's and 2 U/ml IL-2. Peptide-pulsed PBL's are prepared as follows: About 10⁷ cells are centrifuged and resuspended in 100 µl PBS containing 100 µg HER2/neu peptide. After 1 h at 37°C, the cells are irradiated (2000 R), washed once and added to the cultures. When cell densities exceed 10⁶/ml, cells are diluted to 5 x 20 10⁵/ml by centrifugation and resuspended in RPMI 1640 plus 10% pooled human plasma with 2 U/ml IL-2. Every 10 days 25 the cells are pulsed with HER2/neu peptide as described above.

30 Example 7: Construction of Peptide Expression Vector

A. Construction of episomal vector

35 The expression plasmid p8901 is constructed by ligation of oligonucleotide duplexes containing the Kozak consensus sequence, and coding for the appropriate amino acid residues of the mutant antigenic peptide into the unique BamHI site of plasmid p8901 (Bednarek et al., supra, incorporated herein by reference). Plasmid p8901 contains

cells are pelleted and resuspended in 24 ml hygromycin B followed by transfer to 10 ml RPMI/FCS. After 24 h at 37°C el ectrode gap) cells are incubated on ice for 10 min electrode (Bio-Rad Laboratories, Richmond, CA) (0.4 cm V and 960 μ Farad in a Bio-Rad Gene Pulser and Capacitance ml RPMI, and 20 μ g DNA added. After electroporation at 230 ml host cells (e.g. T2 cells) (10⁶) are suspended in 0.5 B. Transfection of episomal expression vectors.

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Sequenase (US Bioc hemicals Corporation, Cleveland, OH). Confirmed by sequencing of the double stranded vector using vector. The orientation of the synthetic transcripts is phosphatase-treated P8901 yields the desired expression of the digested fragment into BamHI digested and alkaline generates a cohesive end compatible with BamHI. Ligation (10 U) and BglIII (20 U) for 8 h. The BglIII digest is extracted, ethanol precipitated, and digested with BamHI polymerase chain reaction product (1 μ g) is phenol reaction is used to amplify the desired duplex. The oligonucleotides with Klenow and DNTP, polymerase chain of oligonucleotides containing the annealed pair complementary overlap. After extracting the annealed pair synthetic oligonucleotides containing 15 bp of The oligonucleotide duplexes are constructed from transfectected human cells.

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promoter) sequences and confers hygromycin B resistance to thymidine 1 promoter and termination (HSV thymidine 1 hpt gene is inserted between the herpes simplex virus propagation of the vector in Escherichia coli. The R. coli (pBRori) and ampicillin-resistance gene (Amp') allow RSV nucleic acid 1 gene. The pBR322 origin of replication RSV origin of replication and a functional segment of the replicating RSV episomal replication that includes both the insertion site contains the SV40 large T polyadenylation leader. The 3' flanking sequence following the BamHI sigma. Stable transfection is afforded by the self-inserter is following elements 5, to the BamHI cloning site: the SV40 enhancer; the Ad2 M₁ promoter, and the Ad2 tripartite leader. The 3', flanking sequence following the BamHI

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containing medium (150 μ g/ml: Sigma Chemical Co., St. Louis, MO) and plated at 1 ml/well in 24-well plates.

We have developed three lines of evidence that SEQ ID NO.:2, the mutant antigenic peptide, is actually the endogenous peptide expressed by tumor cells. First, we have performed HPLC on antigenic peptides expressed and presented by cancer cells and have found a reproducible peak that corresponds to that of SEQ ID NO.:2. Second, as shown in the Figures, we have taken synthetic SEQ ID NO.:2 and presented it on empty T2 cells and have exposed it to lymphocytes that have previously been exposed to HLA-A2⁺ HER2/neu expressing tumors. We have shown that these lymphocytes recognize SEQ ID NO.:2 when presented on T2 cells. Third, with reference to Fig. 8, we have sensitized with SEQ ID NO.:2 "uneducated" ovarian lymphocytes (i.e., that have not yet been stimulated by tumor antigen). These ovarian lymphocytes sensitized with synthetic SEQ ID NO.:2 were challenged with a variety of cells as shown in Fig. 8. Cells included T2 cells presenting SEQ ID NO.:1 and SEQ ID NO.:2; allogeneic HLA-A2⁺/HER2/neu⁺ ovarian tumor cells; and allogeneic HLA-A2⁻ ovarian tumor cells.

The results clearly indicate that the sensitized, uneducated ovarian lymphocytes recognize endogenous tumor (bars 3 and 4 of Fig. 8). SEQ ID NO.: 2 alone (i.e. when presented on T2 cells) will also induce recognition by the SEQ ID NO.: 2-educated lymphocytes (bar 2 of Fig. 8). Thus, since SEQ ID NO.: 2-sensitized lymphocytes can recognize endogenous tumor we believe that SEQ ID NO.:2 must be expressed in endogenous tumor and it is capable of being recognized even though it is expressed in small amounts.

Example 8: Stimulation of CTL formation Using HER2/neu Peptide

This experiment demonstrates that repeated stimulation with isolated HER2/neu antigenic peptide of the

35 NY) excision on a Neubauer hemocytometer. Aliquots of
cells were counted using trypan blue (GIBCO, Grand Island,
Teknika Corp., Durham, NC). After washing twice with HBS,
over a discontinuous 75/100% Ficoll gradient (Organon
resuspended. Lymphocytes and tumor cells were separated
centrifuged at 800xg for 10 minutes and the cell pellet
30 H11, NJ) was added at 1 unit/ml. The samples were
contaminated and heparin sodium (Becton-Dickin, Inc., Cherry
Cell line: Ascites fluid was collected in sterile

25 whereas CTL line #4, was HLA-A2.
3 patients (designated CTL line 1, 2, 3) were HLA-A2+
of both hospitals. The lymphocyte cultures from the first
Boston, MA under approval of the Institutional Review Board
Brigham and Women's Hospital and Beth Israel Hospital,
provided by the Gynecologic Oncology Department of both
time of laparotomy were examined. Specimens were kindly
adenocarcinoma and greater than 250 ml of ascites at the
patients with histologically confirmed ovarian
20 Patients: Four consecutive previously untreated
Materials and Methods:

15 weakly peptide stimulations of the effector cells.
quantitate the level of target cell cytotoxicity after three
standard 4 hour chromium release assays were used to
tumor stimulate populations and unstimulated controls.
tumor associated lymphocyte (TAL) cultures for comparison
used for weekly HLA-A2 restricted peptide stimulation of
product with an intrinsic antigen processing defect was
similar effect. The T2 cell line, a T-cell/B-cell fusion
stimulation with this antigenic peptide would have a
5 experiments, supra). We hypothesized that repeated
that cytotoxicity against autologous tumor (See
therfore, tumor-specific CTL. We have previously shown
in an oligoclonal expansion of peptide-specific and
invention, when presented in the context of HLA-A2, results

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Whittaker, Walkersville, MD) and 10% DMSO (Sigma, St. Louis, MO).

5 TAL Stimulation: After initial separation over a discontinuous Ficoll gradient as described above, tumor cell and lymphocytes were cultured in RPMI 1640/20% FCS (Bio-Whittaker) supplemented with 50U/ml penicillin, 50 μ g/ml streptomycin, 25mM HEPES buffer and 2mM L-glutamine (Bio-Whittaker). Cells were suspended at 5 x 10⁵ cells/ml on solid phase anti-CD3 plates (Orthoclone 10 OKT3: Ortho Pharmaceutical Corp., Raritan, NJ) as described previously and placed in a humidified incubator at 37°C, 5% CO₂. After 24 hours cells were transferred to uncoated flasks (Costar, Cambridge, MA) and supplemented with 50 IU/ml of IL-2 (AMGEN, Thousand Oaks, CA). Cells from all 15 control and experimental groups were counted weekly using trypan blue exclusion and 1 x 10⁷ cells were resuspended. The remainder were cryopreserved. All cells were 20 supplemented with 50 IU/ml of IL-2 every 3 days. The tumor stimulated lymphocytes were treated weekly with autologous tumor cells irradiated with 7500 rads and washed twice prior to co-culture with TAL. Tumor stimulation was 25 carried out at a 10:1 lymphocyte to tumor cell ratio.

Peptide pulsed T2 stimulation was accomplished as follows: 2 x 10⁶ T2 cells were suspended in 100 μ l of GP2 (SEQ ID NO.: 2) (1mg/ml) and incubated at 37° for 1 hour. Cells were then irradiated with 7500 rads, washed twice, 30 re-counted and added to lymphocytes cultures at a 10:1 lymphocyte: T2 ratio. Control group cells were supplemented with only IL-2 (50 IU/ml) every 3 days. All cultures were maintained in parallel under identical conditions.

35 Phenotyping: Tumor cells were incubated with 10 μ l of the anti-HLA-A2 mAbs, BB7.2 and MA2.1 (ATCC, Rockville, MD), at a dilution of 1:10 and with 10 μ l of the anti-

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(expressed as LU₂₀/10⁷ cells). Statistical significance was analyzed with the paired t-test.

5 Antibody Blocking: Chromium release assays were performed as described. After ^{51}Cr labeling and washing of target cells, 10 μl of BB7.2 was added and target cells were incubated at 4°C for 30 minutes prior to plating in 96 well plates.

10 Results

Peptide stimulated TAL show equivalent or increased proliferation over unstimulated controls. In 2 of 4 patients, control TAL which were treated with IL-2 alone failed to expand to therapeutic numbers. In contrast, TAL stimulated with HER2/neu peptide (SEQ ID NO.: 2) showed a 50- 1400 fold expansion in 21 days which was not significantly different from the tumor-stimulated group (data not shown). As expected, the single exception was TAL line #4, the HLA-2 line which did not show a significant increase in proliferation in the HER2/neu peptide-stimulated group compared with unstimulated and tumor stimulated controls. In 1 of 4 lines (TAL#1), control TAL expanded as well as peptide stimulated and tumor stimulated TAL.

25 Peptide stimulated TAL show variable CD4/CD8 ratios. We
have previously shown that cytotoxicity of ovarian TAL is
optimized after three consecutive stimulations with
irradiated autologous tumor cells. G. Peoples et al.,
30 Surgery, 114: 227-234 (1993). Concurrent with the
cytotoxicity assays, all lymphocytes were phenotyped after
the third weekly peptide stimulation. All cells from the
control, tumor-stimulated and peptide-stimulated groups
showed a high level of CD3⁺ cells. Of the twelve lines
35 tested, peptide stimulated TAL ranged from 35-78% CD 8.
There was no significant difference of percent positive CD8

High cell numbers were required to expand the cell lines and to obtain enough cells for each line at an effector:target (E:T) ratio of 40:1. Interestingly, even the HLA-A2⁺ CTL line showed increased killing versus autologous tumor, again suggesting that an HLA allele containing a similar binding motif to HLA-A2 is present and able to present GP2 (S9Q ID NO.: 2). A significant difference was observed in the cytotoxicity of peptide-stimulated effectors when compared with that in HLA-A2⁺ CTLs present and able to bind GP2 (S9Q ID NO.: 2). The peptide-stimulated control cells (17 \pm 7% VR, $1 \pm 2\%$, $p=0.01$). The highest level of cytotoxicity was seen in the tumor-mimulated group adding to the high likelihood that the tumor-associated antigens (TAA) are expressed on the tumor cell surface. The high percentage of CD3⁺ cells and Virtually absence of CD16⁺ cells argues against non-specific NK cell-mediated cytotoxicity which was confirmed and supported by the fact that the tumor cells are CD3⁺ cells.

Peptide stimulate TAL. Using T2 loaded with synthetic peptides as a target cell in chromium release assays, we found that T2/GP2. Using T2 loaded with synthetic peptides as a target cell in chromium release assays, we found that peptide educated TAL to recognize T2/GP2 but not T2 alone or T2 alone. A statistically significant difference was observed in the specific lysins of peptide pulsed T2 versus T2 alone as shown (33 ± 14 vs. 5 ± 5 , $p=0.01$). Curiously, this difference was also noted in the HLA-A2. TAL line which suggests the presence of another HLA allele capable of binding SRG ID NO. 2 (GP2). SRG ID NO. : 4 (GP1), a HRS/neu derived peptide which the appropriate HLA-A2 binding motif (PLTISIISAV), was not recognized by these peptides stimulate TAL.

CELLS in comparing peptide stimulated versus humor stimulated TAL.

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by the lack of cytotoxicity of peptide stimulated CTL vs. K562, and NK sensitive cell line. (data not shown).

Tumor lysis by peptide stimulated CTL is HLA-A2 restricted.

5 HLA-A2 restriction is suggested by the finding that peptide-stimulated CTL had increased cytotoxicity against allogeneic HLA-A2⁺ targets (data not shown). Lastly, the increased cytotoxicity of peptide stimulated CTL could be almost completely blocked with anti-HLA-A2 mAb (hybridoma 10 supernatant containing BB7.2) in the two lines tested. On the other hand, antibody to HER2/neu gene product specific for an epitope distinct from SEQ ID NO. 2 (GP2) showed no such effect.

15 In this experiment, we have shown HER2/neu derived synthetic peptide (e.g., SEQ ID NO.: 2) can generate peptide-specific tumor associated lymphocytes (TALs). More importantly, our experiment shows that cytotoxic TAL can be sensitized with repeated peptide stimulation, and that this 20 sensitization results in an enhanced level of cytotoxicity against autologous and HLA matched allogeneic tumor cells when compared with unstimulated controls. Predictably, tumor-stimulated lymphocytes have the highest level of cytotoxicity against autologous tumor, as multiple TAA are 25 expressed on the cell surface, and thus, the oligoclonal expansion of multiple tumor-specific effector sub-populations should result.

30 The clinical significance of our findings for adoptive immunotherapy are three-fold. First, peptide stimulation may be safer than tumor stimulation in that one can avoid the adoptive transfer of tumor cells to the patient receiving adoptive immunotherapy. In addition, in many cases, adequate numbers of tumor cells are not available for repeated *in vitro* stimulation. Second, the current 35 standard for adoptive immunotherapy involves the adoptive transfer of tumor infiltrating lymphocytes (TIL) which are extracted from solid tumor tissue and expanded in IL-2.

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invention.

It should be understood that the preceding is merely a detailed description of certain preferred embodiments. It therefore should be apparent to those skilled in the art that various modifications and equivalents can be made without departing from the spirit or scope of the invention.

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therapies.

Since peptide-stimulated TAA from ascites fluids show enhanced cytotoxicity against autologous tumor *in vitro*, one might expect that a similar effect would be seen *in vivo*. Lastly, as more becomes known about TAA and alike specific HIA classes I antigen presentation, these antigens and the peptide epitopes derived from them could be exploited to develop polyvalent anti-tumor vaccine and the peptide epitopes derived from them could be exploited to develop polyvalent anti-tumor vaccine.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

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Fragments and Uses
- (iii) NUMBER OF SEQUENCES: 12

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- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

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- (A) APPLICATION NUMBER:
- (B) FILING DATE:
- (C) CLASSIFICATION:

(vii) ATTORNEY/AGENT INFORMATION:

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

Pro Leu Thr Ser Ile Ile Ser Ala Val

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

(ii) MOLECULE TYPE: peptide

(D) TOPOLOGY: linear

(B) TYPE: amino acid

(A) LENGTH: 9 amino acids

(i) SEQUENCE CHARACTERISTICS:

(2) INFORMATION FOR SEQ ID NO:4:

1 5
Xaa Ile Xaa Xaa Xaa Val Xaa Xaa Xaa Leu

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

(ii) MOLECULE TYPE: peptide

(D) TOPOLOGY: linear

(B) TYPE: amino acid

(A) LENGTH: 9 amino acids

(i) SEQUENCE CHARACTERISTICS:

(2) INFORMATION FOR SEQ ID NO:3:

1 5
Ile Ile Ser Ala Val Val Gly Ile Leu

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

(ii) MOLECULE TYPE: peptide

(D) TOPOLOGY: linear

(B) TYPE: amino acid

(A) LENGTH: 9 amino acids

(i) SEQUENCE CHARACTERISTICS:

(2) INFORMATION FOR SEQ ID NO:2:

1 5
Ile Leu Ser Ala Val Val Gly Ile Leu

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

(v) FRAGMENT TYPE: internal

1 5

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Tyr Ile Ser Ala Val Val Gly Ile Leu

1 5

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Phe Ile Ser Ala Val Val Gly Ile Leu

1 5

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ATCATCTCTG CGGTGGTTGG CATTCTG

27

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single

(x) SEQUENCE DESCRIPTION: SEQ ID NO:11:

(ii) MOLECULE TYPE: DNA (genomic)

- (D) TOPOLOGY: linear
- (C) STRANDEDNESS: single
- (B) TYPE: nucleic acid
- (A) LENGTH: 27 base pairs

(i) SEQUENCE CHARACTERISTICS:

(2) INFORMATION FOR SEQ ID NO:11:

ATHATHAGYGCNGTNGTG NATHTCN

27

(x) SEQUENCE DESCRIPTION: SEQ ID NO:10:

(ii) MOLECULE TYPE: DNA (genomic)

- (D) TOPOLOGY: linear
- (C) STRANDEDNESS: single
- (B) TYPE: nucleic acid
- (A) LENGTH: 27 base pairs

(i) SEQUENCE CHARACTERISTICS:

(2) INFORMATION FOR SEQ ID NO:10:

ATHATHCAGCNGTNGTG NATHTTR

27

(x) SEQUENCE DESCRIPTION: SEQ ID NO:9:

(ii) MOLECULE TYPE: DNA (genomic)

- (D) TOPOLOGY: linear
- (C) STRANDEDNESS: single
- (B) TYPE: nucleic acid
- (A) LENGTH: 27 base pairs

(i) SEQUENCE CHARACTERISTICS:

(2) INFORMATION FOR SEQ ID NO:9:

ATHATHAGYGCNGTNGTG NATHTTR

27

(x) SEQUENCE DESCRIPTION: SEQ ID NO:8:

(ii) MOLECULE TYPE: DNA (genomic)

- (D) TOPOLOGY: linear

96

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ATHATHGTGNG CNGTNGTNGG NATHCTN

27

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Phe Leu Pro Ser Asp Tyr Phe Pro Ser Val
1 5 10

1. An isolated peptide either 9 or 10 amino acid residues in length, the isolated peptide including at least a leucine residue at a C-terminal thereof, said peptide capable of engagement with an HLA-A2 binding cleft and presented to at least one T-lymphocyte.

2. The isolated peptide of claim 1, wherein said peptide includes an isoleucine residue at position 2.

3. The isolated peptide of claims 1 or 2, wherein said peptide includes a valine residue at position 6.

4. The isolated peptide of claim 1, wherein said peptide is expressed only in tumor cells.

5. The isolated peptide of claim 3, wherein said peptide is a fragment of an oncogene product.

6. The isolated peptide of claim 5, wherein said oncogene is HHR2/neu.

7. An isolated HHR2/neu mutant peptide of sufficient size and amino acid composition to engage with an HLA-A2 binding cleft.

8. The isolated mutant peptide of claim 7, said peptide having no greater than 10 amino acids and capable of stimulating proliferation of at least one cytotoxic T-lymphocyte when said isolated mutant peptide is engaged with the HLA-A2 binding cleft and presented to at least one T-lymphocyte.

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Claims

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9. The isolated mutant peptide of claim 8, wherein said cytotoxic T-lymphocyte is obtainable from an ovarian tumor.

5 10. The isolated mutant peptide of claim 8, wherein said cytotoxic T-lymphocyte is obtainable from a breast tumor.

11. The isolated mutant peptide of claim 8, wherein said cytotoxic T-lymphocyte is obtainable from a non-small cell lung tumor.

10 12. The isolated mutant peptide of claim 8, wherein said cytotoxic T-lymphocyte is obtainable from a gastric tumor.

15 13. The isolated mutant peptide of claim 8, wherein said cytotoxic T-lymphocyte is obtainable from a pancreatic tumor.

14. The isolated mutant peptide of claim 8, wherein said cytotoxic T-lymphocyte is obtainable from a colon tumor.

20 15. The isolated mutant peptide of claim 8, wherein said cytotoxic T-lymphocyte is obtainable from peripheral blood lymphocytes.

25 16. The isolated mutant peptide of claim 7 having an amino acid sequence comprising SEQ ID NO.:1 and functional equivalents thereof.

30 17. The isolated peptide of claim 1, selected from the group consisting of SEQ ID NOS. 2, 3, 5 and 6.

35 18. An isolated nucleic acid sequence capable of encoding a peptide fragment either 9 or 10 amino acid residues in length, said peptide fragment including at least a leucine residue at a C-terminus thereof, said peptide fragment capable of engaging with an HLA-A2 binding cleft and capable of stimulating proliferation of at least one

5. The cytotoxic T-lymphocyte when said peptide is engaged with the cytotoxic T-lymphocyte when said peptide binds to at least one T-lymphocyte.

19. The isolated nucleic acid sequence of claim 18, wherein said encoded peptide further includes an isolated peptide residue at position 2.

20. The isolated nucleic acid of claims 18 or 19, wherein said encoded peptide further includes a valine residue at position 6.

21. The isolated nucleic acid sequence of claim 18, said a nucleic acid sequence capable of encoding a mutant HRR2/neu peptide fragment.

22. The isolated nucleic acid sequence of claim 18, said a nucleic acid sequence capable of encoding a peptide nucleic acid sequence of claim 18, said

20. The isolated nucleic acid sequence of claim 18 or 19, wherein said encoded peptide further includes a valine residue at position 6.

21. The isolated nucleic acid sequence of claim 18, said a nucleic acid sequence capable of encoding a mutant HRR2/neu peptide fragment.

22. The isolated nucleic acid sequence of claim 18, said a nucleic acid sequence capable of encoding a peptide nucleic acid sequence of claim 18, said

23. A recombinant vector comprising said nucleic acid of claim 18.

24. A host cell containing the recombinant vector of claim claim 18.

25. An isolated population of cytotoxic T-lymphocytes capable of proliferating in the presence of the isolated peptide of claims 1 or 7 when said isolated peptide is presented in an HLA-A2 binding cleft.

26. The isolated cytotoxic T-lymphocytes being obtainable from an ovarian tumor.

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27. The isolated cytotoxic T-lymphocytes of claim 25, said cytotoxic T-lymphocytes being obtainable from a breast tumor.

5 28. The isolated cytotoxic T-lymphocytes of claim 25, said cytotoxic T-lymphocytes being obtainable from a non-small-cell lung tumor.

10 29. The isolated cytotoxic T-lymphocytes of claim 25, said cytotoxic T-lymphocytes being obtainable from a gastric tumor.

15 30. The isolated cytotoxic T-lymphocytes of claim 25, said cytotoxic T-lymphocytes obtainable from a pancreatic tumor.

15 31. The isolated cytotoxic T-lymphocytes of claim 25 said cytotoxic T-lymphocytes obtainable from a colon tumor.

20 32. The isolated cytotoxic T-lymphocytes of claim 25, said cytotoxic T-lymphocytes obtainable from peripheral blood lymphocytes.

25 33. A method of stimulating proliferation of tumor-specific cytotoxic T-lymphocytes, comprising the steps of:

obtaining a population of T-lymphocytes from a sample suspected of containing said lymphocytes;

contacting said T-lymphocytes with the isolated peptide of claims 1 or 7 under conditions sufficient for tumor-specific cytotoxic T-lymphocytes to proliferate.

30 34. The method of claim 33, wherein the step of contacting comprises contacting a population of tumor infiltrating or tumor-associated T-lymphocytes with said peptide under conditions sufficient for presentation of said peptide to said T-lymphocytes, the peptide engaged within an HLA-A2 binding cleft.

35. The method of claim 34, wherein the step of contacting lymphocytes containing a population of peripheral blood lymphocytes with said peptide under conditions sufficient to said peptide engaged with an HLA-A2 binding lymphocyte, the peptide presentation of said peptide to said lymphocytes, the amount of the isolated peptide of claims 1 or 7.

36. A composition comprising a therapeutic peptide effective for preventing a disease or condition, the peptide being a peptide containing a HLA-A2 binding lymphocyte, the peptide being isolated peptide of said peptide presentation of said peptide to said lymphocyte, the peptide being isolated peptide of said peptide presentation of said peptide to said lymphocyte.

37. The composition of claim 36, further comprising an immunoadjuvant.

38. The composition of claim 36, further comprising a carrier.

39. The composition of claim 38, wherein the carrier is a cellulose.

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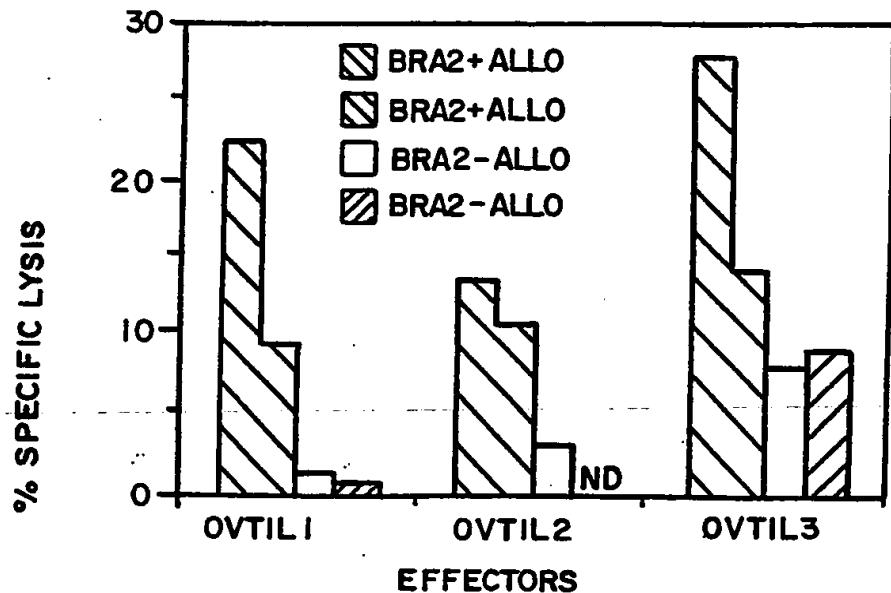


FIG. 1

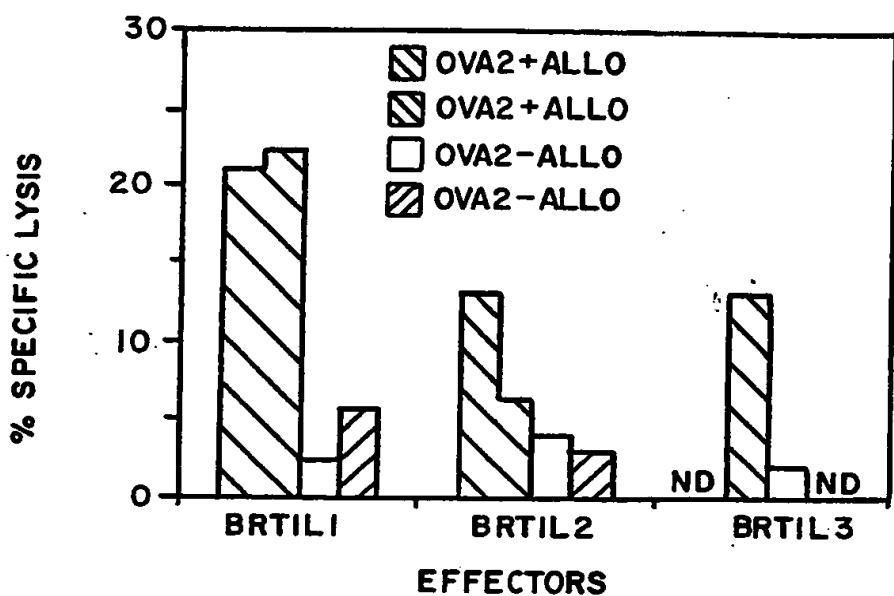


FIG. 2

SUBSTITUTE SHEET (RULE 26)

FIG. 4

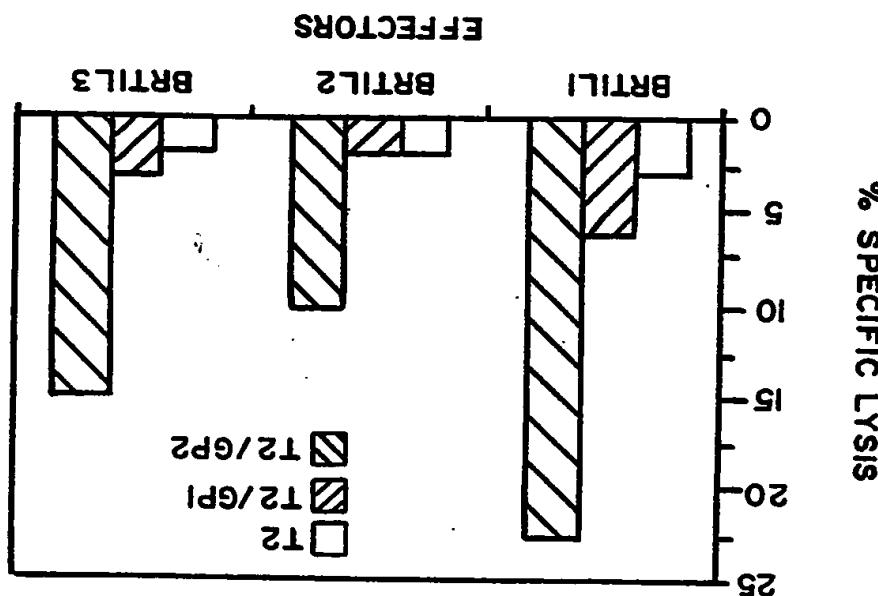
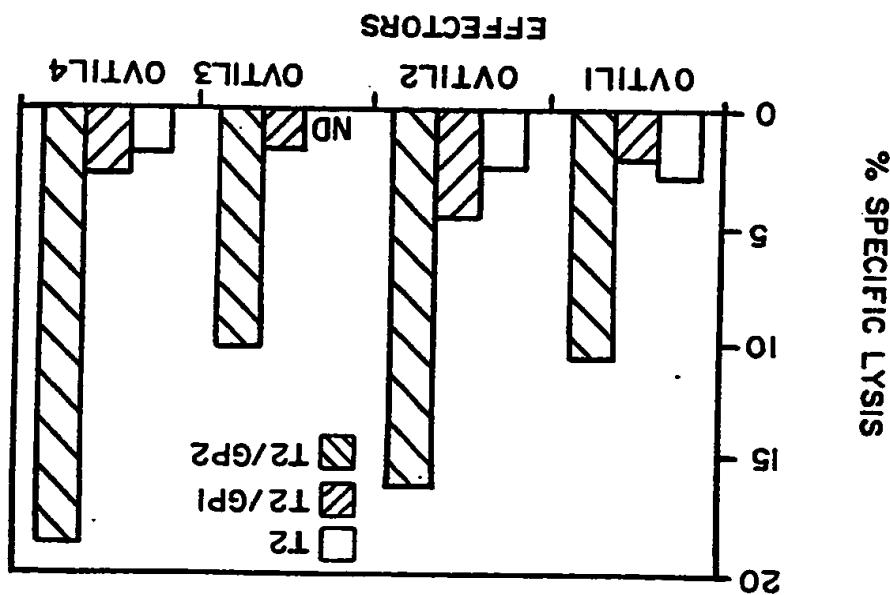


FIG. 3



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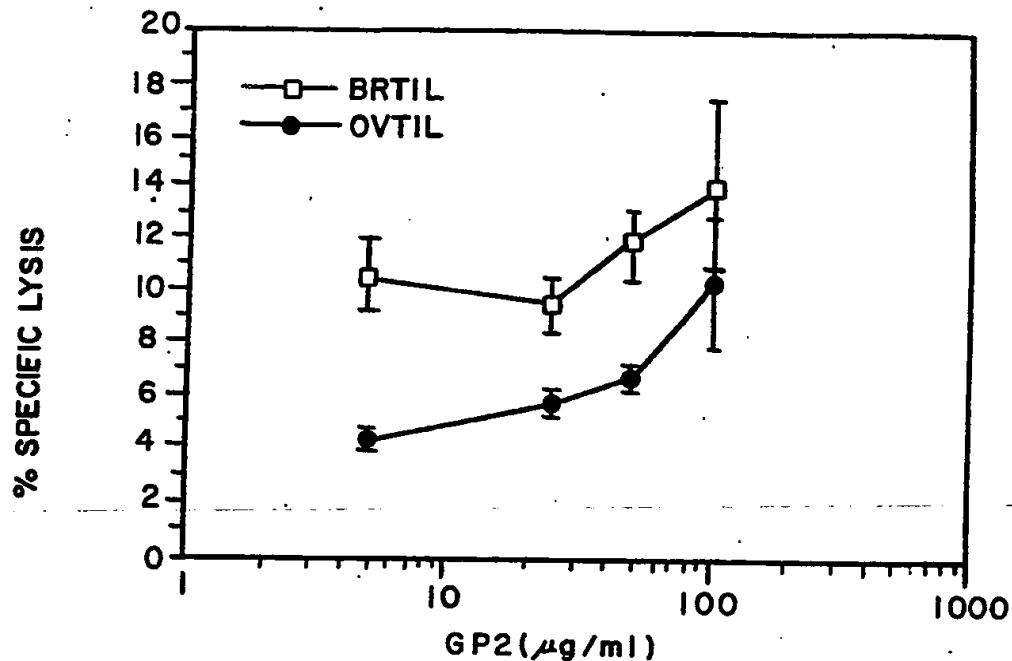


FIG. 5

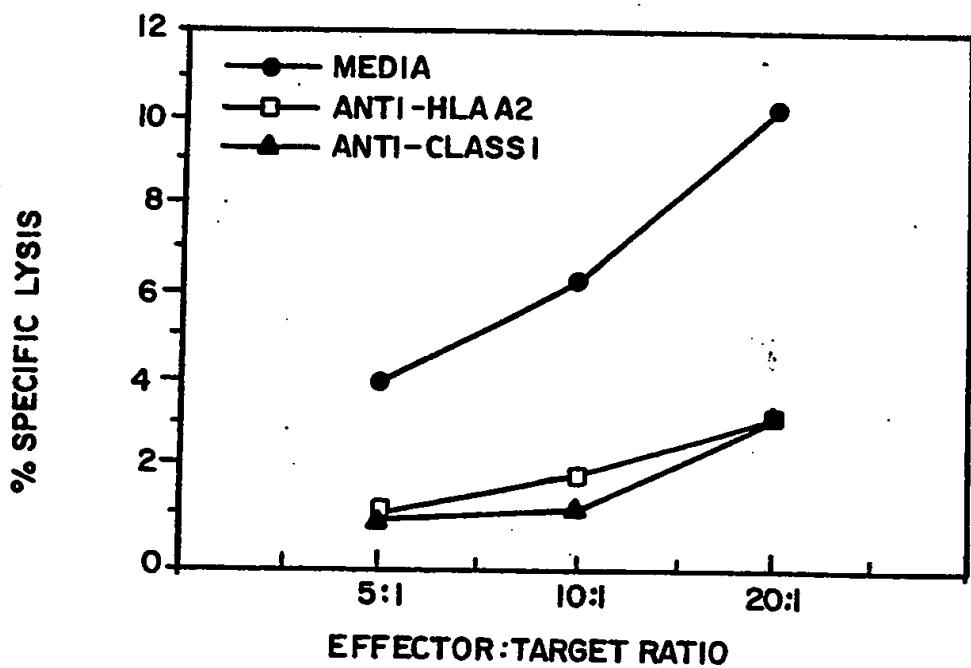


FIG. 6

SUBSTITUTE SHEET (RULE 26)

FIG. 8

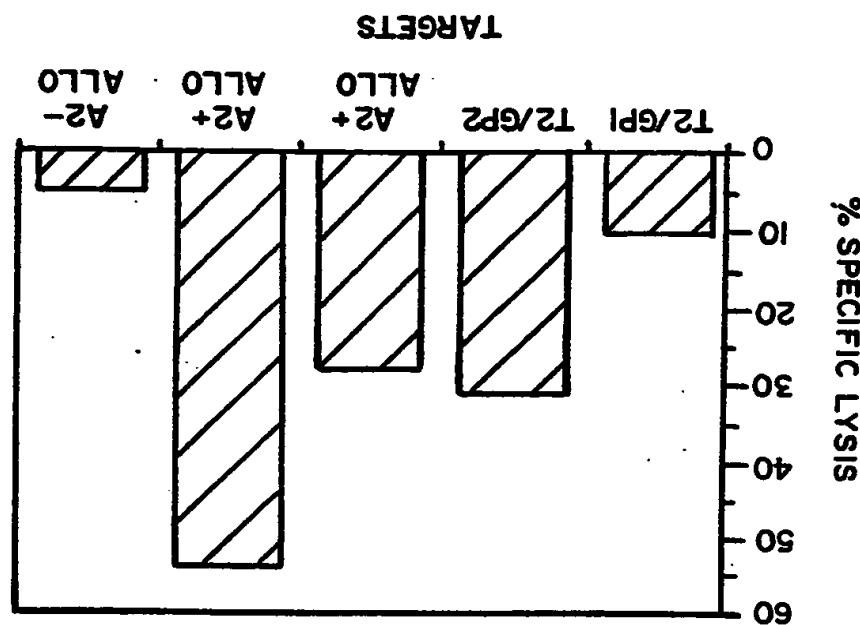
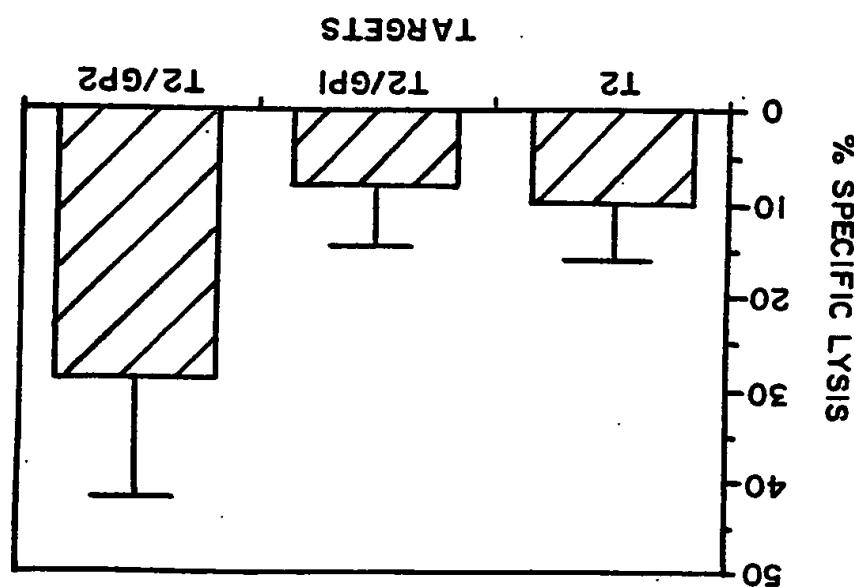


FIG. 7



INTERNATIONAL SEARCH REPORT

Intern: Application No
PCT/US 95/01672A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/71 A61K38/08 C12N15/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF IMMUNOLOGY., vol.152, no.1, 1 January 1994, BALTIMORE US pages 163 - 175 K C PARKER ET AL. 'Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chain' see table 1 ---- -/-	1-3

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

* Special categories of cited documents:

- 'A' document defining the general state of the art which is not considered to be of particular relevance
- 'B' earlier document but published on or after the international filing date
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Date of the actual completion of the international search

13 June 1995

Date of mailing of the international search report

21-06-1995

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Masturz, P

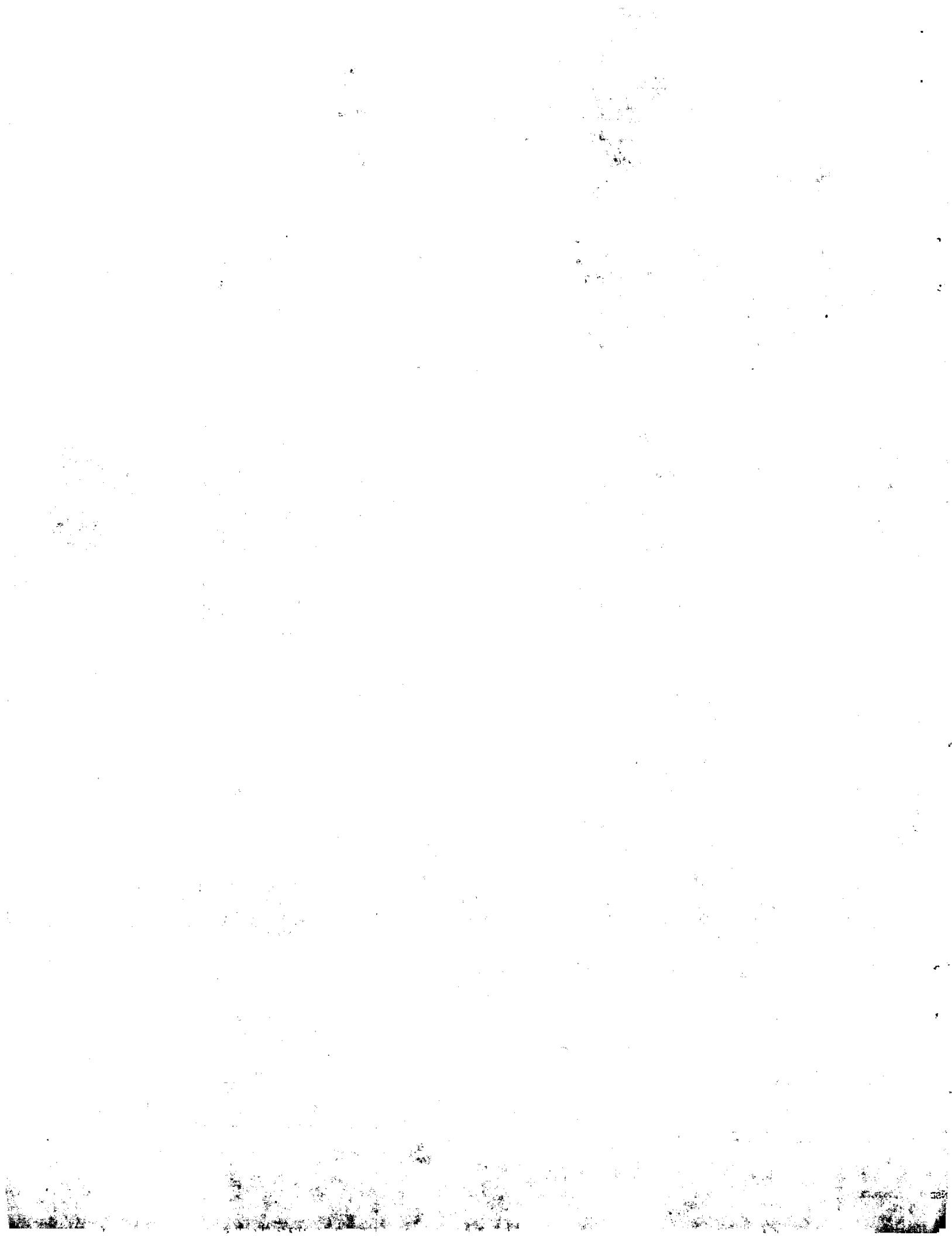
Category	Classification of document, with indication, where appropriate, of the relevant passage(s)	Reference to claim No.
PCT/US 95/01672	(Classification) DOCUMENTS CONSIDERED TO BE RELEVANT	
X	INTERNATIONAL SEARCH REPORT	
X	HUMAN IMMUNOLOGY, V 1.37, no.4, December 1993, NEW YORK Pages 252 - 258 S Y SAMUA ET AL. "Recognition by HLA-A2-restricted cytotoxic T lymphocytes of endogenous self-synthetic peptides and exogenous peptides, influenced by a matrix protein" see claim 2	
X	J IMMUNOTHERAPY WITH EMPHASIS ON TUMOR IMMUNOLOGY, V 1.14, no.2, August 1993, NEW YORK Pages 121 - 126 H W NIJMAN ET AL. "Characterization of a cytotoxic T lymphocytes epitopes of a self-protein, p53, and a non-self-protein, infuzenza matrix; relationship between major histocompatibility complex peptide binding affinity and immune responsiveness to peptides, see table 1"	I-3,25
X	JOURNAL OF IMMUNOLOGY., Vol. 149, no.11, 1 December 1992, BALTIMORE US Pages 3580 - 3587 K C PARKER ET AL. "Sequence motifs important for peptide binding to the human MHC Class I molecule, HLA-A2, see the whole document"	I-3
P,X	CANCER RESEARCH, Vol. 54, no.13, 1 July 1994, PHILADELPHIA, PA, USA Pages 3387 - 3390 I YOSHINO ET AL. "HER2/neu-derived peptides are shared antigens among human non-small cell lung cancer and ovarian cancer, see the whole document"	I-39

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 95/01672

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA., vol.92, no.2, 17 January 1995, WASHINGTON US pages 432 - 436 G E PEOPLES ET AL. 'Breast and ovarian cancer-specific cytotoxic T lymphocytes recognize the same HER2/neu-derived peptide' see the whole document -----	1-39



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/01672

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
Remark : Although claims 33-35 refer to a method of treatment of the human body, the search was carried out and based on the alleged effects of the products.
2. Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORTInternat'l Application No
PCT/US 95/01672

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9008160	26-07-90	NONE	

Form PCT/ISA/218 (patent family annex) (July 1992)

